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ASSESSMENT OF ENVIRONMENTAL CONTAMINANTS' NEUROTOXICITY:

IN VITRO AND *IN VIVO* EXPERIMENTAL STUDIES

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ABSTRACT

Accumulating evidence points to environmental contaminants as possible causes of neuronal damage in developing organisms. A prerequisite to prevention is the recognition of a chemical's harmful effects during development. The knowledge that an environmental contaminant is neurotoxic can prompt efforts to restrict its use and to limit the exposure. Many studies on environmental toxicants have been performed so far, but the knowledge available on the effects of exposures at low, environmentally relevant doses, and on cell-specific mechanisms of action is still limited. The work included in this thesis is based on an experimental strategy including *in vitro* studies and behavioral analyses aimed at investigating the potential neurotoxic effects of selected food contaminants, such as polychlorinated biphenyls (PCBs), methylmercury (MeHg) and perfluorinated chemicals (PFCs). Using *in vitro* models, we found that MeHg and PCBs cause cell death in the hippocampal cell line HT22 via a parallel activation of calpains and lysosomal proteases, with no involvement of caspases. Oxidative stress does not play a major role in PCBs toxicity, opposite to MeHg and co-exposure to PCBs and MeHg show mostly antagonistic interactions. We have also investigated the effects of non-dioxin like (NDL)-PCBs 153 and 180 and MeHg on primary cultures of rat neural stem cells (NSCs). Both PCBs promote neuronal differentiation and decrease NSCs' proliferation by repressing Notch signaling. Conversely, exposure to MeHg inhibits neuronal differentiation and promotes the proliferation of NSCs by stimulating Notch signaling. The effects on differentiation were confirmed by the changes in the number of cells showing spontaneous Ca^{2+} activity following the exposure to PCBs or MeHg. Combined exposures to PCBs and MeHg resulted in antagonistic effects on spontaneous neuronal differentiation, but induced apoptosis, which was not observed with single exposure to either chemical. We used the same model for investigating the effects of nanomolar concentrations of perfluorooctane sulfonate (PFOS), and we found that PFOS stimulates neuronal and oligodendrocytic differentiation in a dose-dependent manner by upregulating PPARγ and the downstream gene, mitochondrial uncoupling protein 2 (UCP2). Importantly, the effects were confirmed in mouse neonatal brains after prenatal exposure to PFOS, where we found an increased expression of PPARγ and the downstream gene UCP3. We then investigated the effects of PFCs *in vivo* by assessing the behavioral alterations induced by *in utero* exposure to PFOS or perfluorooctanoic acid (PFOA). We investigated the motor function, circadian activity, and emotion-related behavior. Exposure to PFOS results in decreased locomotion in a novel environment and reduced muscle strength only in male offspring. Prenatal exposure to PFOA is associated with changes in exploratory behavior in both male and female offspring, and increased home cage global activity only in males. In conclusion, our studies show that even very low doses (in the nanomolar range) of selected food contaminants, and the effects found *in vitro* are consistent with the results from *in vivo* exposure. Therefore, a combined approach with both *in vitro* and *in vivo* experimental models is most valuable for developmental neurotoxicity testing.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to in the text by their Roman numbers:

I. Tofighi R, Johansson C, Goldoni M, Ibrahim WN, Gogvadze V, Mutti A, Ceccatelli S.

Hippocampal neurons exposed to the environmental contaminants methylmercury and polychlorinated biphenyls undergo cell death via parallel activation of calpains and lysosomal proteases

Neurotox Res. 2011;19(1):183-94

II. Tofighi R*, Wan Ibrahim WN*, Rebellato P, Andersson PL, Uhlén P, Ceccatelli S.

Non-dioxin-like polychlorinated biphenyls interfere with neuronal differentiation of embryonic neural stem cells

Toxicol Sci. 2011;124(1):192-201

III. Wan Ibrahim WN, Tofighi R, Onishchenko N, Rebellato P, Uhlén P, Ceccatelli S.

PFOS induces neuronal and oligodendrocytic differentiation in neural stem cells and alters the expression of PPARγ *in vitro* and *in vivo*

Submitted to Toxicology Applied Pharmacology

IV. Onishchenko N, Fischer C, Wan Ibrahim WN, Negri S, Spulber S, Cottica D, Ceccatelli S.

Prenatal exposure to PFOS or PFOA alters motor function in mice in a sex-related manner

Neurotox Res. 2011;19(3):452-61

*equal contribution

CONTENTS

LIST OF ABBREVIATIONS

AhR	Aryl hydrocarbon receptor
ANOVA	Analysis of Variance
AO	Acridine orange
ATP	Adenosine triphosphate
BBB	Blood brain barrier
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
cDNA	Complementary DNA
CH3-	Methyl group
CNPase	2',3'-Cyclic-nucleotide 3'-phosphodiesterase
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CO_2	Carbon dioxide
DEPC	Diethylpyrocarbonate
DL	Dioxin-like
DMSO	Dimethyl sulfoxide
DPF	days post fertilization
DNA	Deoxyribonucleic acid
E	Embryonic day
EDCs	Endocrine disrupting compounds
EDU	5-ethynyl-2′-deoxyuridine
EFSA	European Food Safety Authority
EGF	Epidermal growth factor
FCS	Fetal calf serum
GD	Gestation day
GFAP	Glial fibrillary acidic protein
HBSS	Hanks' Balanced Salt Solution
HPF	Hour post fertilization
HPLC-MS	High Performance Liquid Chromatography Mass Spectrometry
IQ	Intelligence quotient
LIF	Leukemia inhibitory factor
LP	Left primer
MeHg	Methylmercury
MnTBAP	Mn(III)tetrakis (4-benzoic acid) porphyrin
mRNA	Messenger ribonucleic acid
µM	Micromolar
NDL	Nondioxin-like
HNP	Human neural progenitor
NICD	Notch intracellular domain
nM	Nanomolar
NSCs	Neural stem cells
OMM	Outer mitochondrial membrane
PBS	Phosphate-buffered saline
PCBs	Polychlorinated biphenyls
PCDDs	Polychlorinated dibenzo-p-dioxins

PCNA	Proliferating cell nuclear antigen
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PF	Paraformaldehyde
PFCs	Perfluorinated chemicals
PFOA	Perfluorooctanoic acid
PFOS	Perfluorooctanesulfonic acid
POPs	Persistent organic pollutants
PPARs	Peroxisome proliferator-activated receptors
Ppm	Parts per million
PPREs	Peroxisome proliferator hormone response elements
PS	Phosphatidylserine
RGZ	Rosiglitazone
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RP	Right primer
RT	Room temperature
SOD	Superoxide dismutase
TMRE	Tetramethylrhodamine ethyl ester
Tuj 1	Neuron-specific class III beta tubulin
VMR	Visual motor response

1 INTRODUCTION

1.1 DEVELOPMENTAL NEUROTOXICITY

Neurotoxicity is defined as the study of the adverse effects induced by exogenous or endogenous factors, including biological, chemical or physical agents, on the nervous system (Philbert *et al.*, 2000; Tilson *et al.*, 1995). The developing central nervous system (CNS) is a 'work under progress' organ, constantly undergoing remodeling, where active proliferation, differentiation, migration, synaptogenesis, and circuitry establishment take place within a tightly controlled time frame. Each developmental stage is to be completed according to a tight schedule. This creates 'windows of susceptibility', *i.e.* the nature and the extent of neurotoxic effects are strongly dependent on the timing of the insults.

The periods of embryonic, fetal, and infant development are remarkably susceptible to environmental hazards (Rodier, 1995). The developing brain is particularly vulnerable to toxic insults as compared to the adult brain also because of the lack of functional barriers. The placenta offers only a partial protection against chemical exposures, and the human blood brain barrier (BBB) is fully developed no sooner than 6 month after birth (Adinolfi, 1985; Risau & Wolburg, 1990). In addition, most of the organic pollutants bind to fatty acids and proteins and can therefore be excreted in breast milk, thereby posing a risk of exposure to newborns and infants (Figure 1.1) (Björnberg *et al.*, 2005; Shen *et al.*, 2012; Sundström *et al.*, 2011). Moreover, developing fetuses, newborns and children may have greater exposures than adults on a unit weight basis, as they have reduced ability to detoxify exogenous substances.

The consequences of a developmental damage may not necessarily be apparent until a critical age, when a neurodevelopmental defect may be unmasked or precipitated by a subsequent exposure to other insults. This delay in the appearance of a neuronal injury is often referred to as 'silent damage' (Reuhl, 1991). Among the most common neurodevelopmental disorders are learning and memory problems, sensory deficits, developmental delays, and cerebral palsy (Boyle *et al.*, 1994), which can all cause lifelong disabilities. Moreover, increasing evidence pointing to links between chemical pollutants, including food contaminants, and neurodevelopmental disorders (see (Grandjean & Landrigan, 2006) for review) makes the study of developmental neurotoxicity pivotal for formulating effective guidelines and strategies to limit the exposure to hazardous chemicals.

1.2 ENVIRONMENTAL CONTAMINANTS

According to a report by the United States Environmental Protection Agency (U.S. EPA) (see (U.S. EPA, 1998)), a large number of chemicals in commerce do not have basic toxicity data publicly available and about 80% have no information about developmental or pediatric toxicity. Lack of toxicity information may result in improper control and handling of these chemicals, which could lead to accidental or deliberate release in the environment. Since a wide range of chemicals have been

shown to affect the developing nervous system, the presence of a large number of yet untested chemicals in the environment becomes a major concern.

In this thesis, we investigated commonly found food contaminants namely polychlorinated biphenyls(PCBs), methylmercury (MeHg) and perfluorinated chemicals (PFCs), as there is increasing concern because of their persistence and global spreading. These chemicals undergo bio-accumulation (the levels in exposed organisms increase with continued exposure) and bio-magnification (the levels increase with the trophic level), and pose a threat to the developing nervous system by exposure through the diet of pregnant or breastfeeding females. The study of the toxic effects of these contaminants is relevant as they still pose a threat to human health after being phased out because of continuous exposure, albeit at low level.

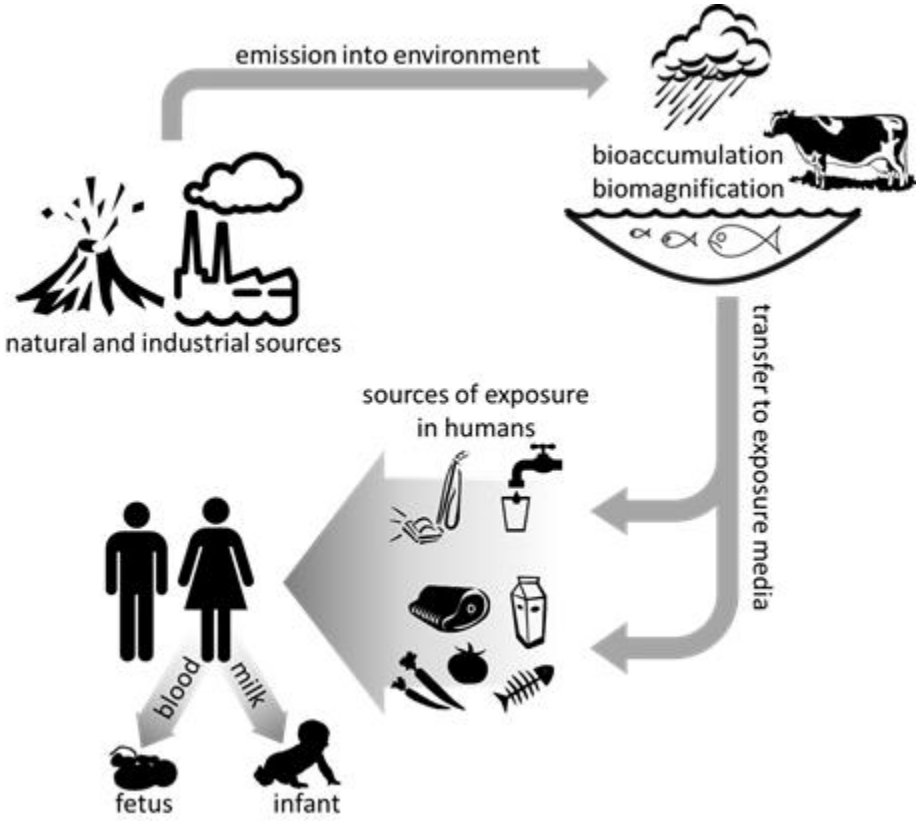

Figure 1.1 A simplified illustration of emission of pollutants into the environment. Bio-accumulated pollutants are bio-magnified in the food chain, and transferred to exposure media. Women that get exposed to pollutants can transfer them to the fetus through blood, and to the infant through breast milk.

1.2.1 Persistent organic pollutants (POPs)

POPs have been defined as "organic chemical substances, which possess a particular combination of physical and chemical properties such that, once released into the environment, they remain intact for exceptionally long periods of time; become widely distributed throughout the environment as a result of natural processes involving soil, water and, most notably, air; accumulate in the fatty tissue of living organisms including humans, get biomagnified and are toxic to both humans and wildlife"

(Stockholm Convention, 2001, amended 2009). Initially, twelve POPs were recognized as causing adverse effects in humans and the ecosystem. In 2009, the Convention listed 9 additional chemicals. The list of these chemical will continue to increase as more chemicals are being produced every year.

The toxic effects of POPs can include cancer, allergies and hypersensitivity, damage to the central and peripheral nervous systems, reproductive disorders, and disruption of the immune system. Some POPs are also considered to be endocrine disrupting chemicals (EDCs) because they interfere with the function of the endocrine axes in exposed individuals, as well as in their offspring (Frye *et al.*, 2012). Humans are mainly exposed to EDCs through ingestion of contaminated food or water, inhalation of polluted air or from dermal exposure (Baillie-Hamilton, 2002; Pelletier *et al.*, 2003). There is a complexity in the response to exposure to EDCs depending on timing, dose, and sex. There is evidence that EDCs can exhibit adverse effects at concentrations far below those currently being tested in toxicological studies, contradicting the assumption that dose–response relationships are monotonic (Welshons *et al.*, 2003; Welshons *et al.*, 2006). In addition to the dose levels, the effects of exposure may depend on gender, which partly may be explained by the fact that some EDCs display estrogenic effects, others anti-estrogenic and/or anti-androgenic effects (Bonefeld-Jørgensen *et al.*, 2001; Hansen, 1998). The mode of action of EDCs is not limited to the direct interaction with hormone receptors. Other mechanisms include inhibition of hormone synthesis, transport, or metabolism and activation of receptors. Data from human and experimental animal studies clearly indicate that prenatal exposure to certain EDCs can have adverse effects on neurodevelopment, neuroendocrine function, as well as on behavior (Parent *et al.*, 2011; Schell & Gallo, 2010). Among the POPs classified as EDCs are organophosphate insecticides, polybrominated diphenyl ethers (PBDEs), PCBs and PFCs.

The negative effects of POPs on the nervous system may be either direct (e.g. by interfering with fundamental cellular processes, such as proliferation, differentiation and cell death), or indirect, by disrupting the endocrine system during development. Here we focus on the direct neurotoxic effects of POPs. Thus, we tested PCBs and PFCs in different experimental models relevant for understanding the mechanisms of neurotoxicity.

1.2.1.1 Polychlorinated biphenyls (PCBs)

PCBs are man-made chemicals and comprise a family of 209 possible congeners. The congeners differ from one another in number and position of the chlorine atoms in the two benzene rings (Figure1.2). The individual PCBs have different chemical characteristics, which influence their uptake, metabolism and accumulation in living organisms. PCBs are fat-soluble and have long half-lives which are estimated to be 10-15 years in humans (Ritter *et al.*, 2011). PCBs fall into two distinct categories. One category consists of rigid, coplanar congeners, which are jointly referred to as “dioxin-like” (DL) because of the structural and toxicity similarities to polychlorinated dibenzo-p-dioxins. The DL-PCBs bind to the aryl hydrocarbon receptor (AhR) transcription factor. The other PCBs, non-coplanar congeners are referred to as “non-dioxin-like” (NDL) and are considered less toxic, but are more abundant in the environment. PCBs

were widely used as coolants, lubricants, and dielectric insulators in electrical components. During their manufacture and use, PCBs were released into the atmosphere via industrial emissions, weathering of PCB-containing materials, and incineration of PCB-containing products. In the 1970's, the production of PCBs was phased out in the U.S. and an international ban on production of these compounds was enacted at the Stockholm Convention on Persistent Organic Pollutants in 2001. However, PCBs continue to be released into the environment from leakage of defunct equipment and leakage from landfills and from previously contaminated soil and sediments. Despite it is more than 30 years PCBs were phased out, studies indicate that NDL PCBs currently predominate in biological and environmental samples, and PCB 153 has been identified as a major contributor to the total PCB burden in humans (Agudo *et al.*, 2009; Axelrad *et al.*, 2009; Longnecker *et al.*, 2003; Moon *et al.*, 2009). Moreover, a recent pilot bio-monitoring study investigating circulating metals and POPs concentrations in Canadian and non-Canadian born primiparous women showed that PCB 153 and PCB 180 are among pollutants that were detected in more than 90% of the samples (Foster *et al.*, 2012). PCBs have been found in both ocean and fresh-water fish (Gewurtz *et al.*, 2011; Madenjian *et al.*, 2009; Mezzetta *et al.*, 2011). The highest content of PCBs in one dietary study was found in dairy products, meat, and fish (Zuccato *et al.*, 1999). Hence, food may be considered as the main source of human exposure to PCBs.

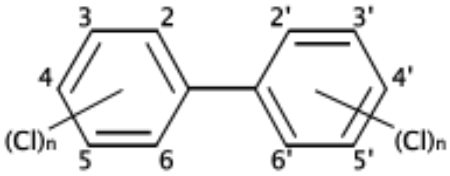

Figure 1.2 Two-dimensional depiction of the generic PCB molecule structure. The total number and the positions of chlorine substitutes varies among congeners and accounts for the different biological effects.

The PCBs are known developmental neurotoxicants, and may affect a variety of neuropsychological functions in children, including general cognition, visual-spatial function, memory, attention, executive and motor functions (Boucher *et al.*, 2009; Schantz *et al.*, 2003). Exposure during development may occur through blood transfer from pregnant women or through excretion of PCBs in breast milk to neonates and young children (Forns *et al.*, 2012; Gascon *et al.*, 2012; Weldon *et al.*, 2011) (see also Figure 1.1). Children exposed *in utero* to PCBs via fish consumption exhibited problems with intellectual functioning (Jacobson *et al.*, 1985). A study on mother-infant pairs in Germany revealed that *in utero* exposure to PCBs affected the mental and motor neurological development of children (Walkowiak *et al.*, 2001). Exposed children also have lower intelligence quotient (IQ) levels than children who are not exposed *in utero* and have increased rates of hyperactivity, with both problems persisting as they grow (Chen *et al.*, 1992; Chen *et al.*, 1994). A study on Inuit preschoolers after both prenatal and postnatal PCBs exposure revealed that exposure to PCB 153 was associated with increased states of unhappiness and anxiety (Plusquellec *et al.*, 2010). The PCBs congeners 2,2'-dichlorobiphenyl, a putative neurotoxic congener, and 3,3',4,4',5-pentachlorobiphenyl have been shown to alter calcium homeostasis in rat cerebellar granule cells (Kodavanti *et al.*, 1993). An investigation to elucidate the mechanism of toxicity induced by PCB mixtures (Aroclors) on primary cultures of cortical neuron revealed that Aroclors induced apoptosis via Bcl-2 family

proteins and caspase-3 proteases (Sanchez-Alonso *et al.*, 2004). Treatment of primary human neural progenitor (HNP) with PCB 118 but not with PCB 126 has been shown to increase formation of oligodendrocytes. The same study showed that the effect of PCBs was congener specific and exposure to PCB 118 mimics T3 action and interferes with thyroid hormone signaling (Fritsche *et al.*, 2005).

1.2.1.2 Perfluorinated chemicals (PFCs)

PFCs are a family of substances that are very stable due to the replacement of carbon-bound hydrogen by fluorine atoms (Figure 1.3) (see (Fromme *et al.*, 2009)). PFCs are extremely persistent and have substantial bio-accumulating and bio-magnifying properties, although they do not follow the typical pattern of other POPs by partitioning into fatty tissues. Instead, they bind to proteins in blood and liver. PFCs elimination half-life is estimated approximately 4-5 years in humans and persistence of these compounds have raised concerns about the potential adverse impact on human health (Olsen *et al.*, 2007). Owing to the both lipid- and water-repellent properties of these chemicals, they have been extensively used as surface-active agents. Their properties make them suitable even for applications involving high-temperatures or contact with strong acids or bases. They are used in a wide variety of products such as in textiles and leather, metal plating, food packaging, and firefighting foams. The voluntary phase-out of PFCs production by the major producer in the U.S. between 2000 and 2002 has led to a significant reduction in the use of PFCs-related substances. However, they are still produced in some countries and there is evidence that they continue to be used. The presence of PFCs in a wide variety of arctic biota, far from anthropogenic sources, shows the capacity of PFCs to undergo long-range transport (Butt *et al.*, 2010). As PFCs-related substances can move in the atmosphere to locations far from their sources, measures taken by single countries or groups of countries are not sufficient to abate the pollution caused by them. Due to the harmful persistent organic pollutant properties and risks related to possible continuing production and use, global action is warranted to eliminate the pollution caused by PFCs.

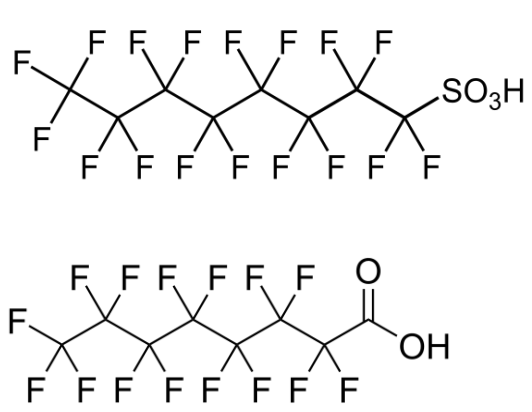

Figure 1.3 Two-dimensional depiction of PFOS (top) and PFOA (bottom) molecules. Although related structurally and sharing the industrial applications, the biological effects vary considerably between the two compounds.

Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) are two most common chemicals found in different biota. The main exposure route of PFOS is believed to be through consumption of contaminated food, such as fish, aquatic invertebrates, marine mammals (Quinete *et al.*, 2009), and other composite food

(Zhang *et al.*, 2010), as well as by contaminated drinking water (Quinete *et al.*, 2009). Developing nervous system get exposed to PFCs through blood from mothers (see also Figure 1.1) (Apelberg *et al.*, 2007; Jensen & Leffers, 2008) and breast milk (Sundström *et al.*, 2011). In exposed mammals PFOS leaves the bloodstream and enters most tissues in a dose-dependent manner, especially liver, bone marrow, skin and muscle, with the highest levels detected in liver (Bogdanska *et al.*, 2011). A cross-sectional study has shown an association between the level of PFCs and children's impulsivity (Gump *et al.*, 2011). Developmental toxicity studies on the effects of PFOS in rodents have revealed a reduction of fetal weight, reduced neonatal survival and also impairments in behavioral tests (Johansson *et al.*, 2008; Lau *et al.*, 2004). Neonatal exposure to PFOS or PFOA in mice induces changes in proteins, which are important for neuronal growth and synaptogenesis in the developing brain (Johansson *et al.*, 2009). PFOS inhibits neurite growth and dramatically suppresses synaptogenesis due to abnormal regulation of calcium in the hippocampus (Liao *et al.*, 2008). PFOS was also found to disturb calcium signaling transduction in the rat CNS after gestational and lactational exposure (Liu *et al.*, 2010; Liu *et al.*, 2010). These findings suggest the possible role of calcium in PFOS and PFOA induced toxic effects on the developing CNS in rodents. These compounds also have shown prominent toxicity effects *in vitro*. Slotkin and colleagues have reported that PFOS influences differentiation of PC12 cells by promoting differentiation towards acetylcholine phenotype rather than the dopamine phenotype (Slotkin *et al.*, 2008). A recent study has shown that PFOS exposure of human microvascular endothelial cells, which are the major components of the BBB, can trigger the "opening" of tight junctions through the PI3K signaling pathway (Wang *et al.*, 2011). Exposure of Syrian Hamster Embryo cells has revealed the transforming potential of PFOS in parallel with an increased expression of peroxisome proliferator-activated receptor (PPARs) genes (Jacquet *et al.*, 2012).

1.2.2 Methylmercury (MeHg)

MeHg became a prototypical example of a dietary environmental contaminant after the unfortunate incidents in Minamata and Niigata in Japan in 1950's and 1960's. The pollution at both locations was massive and lasted for several years before it was recognized officially. The symptoms of the affected children in most cases were associated with large and frequent consumption of fish from the contaminated waters. Although all fish contain at least small amounts of MeHg, fish and shellfish from Minamata and Niigata had up to 40 ppm of MeHg (Harada, 1995), well above the average MeHg concentration found in fish from most western countries, including the U.S. (less than 0.5 ppm) (IPCS, 1990). In the following years, there have been reports of prenatal and postnatal MeHg poisoning elsewhere in the world from sources other than fish. These include MeHg treated grain in Iraq (Amin-Zaki *et al.*, 1976) and consumption of MeHg contaminated pork in New Mexico (Davis *et al.*, 1994). Inorganic mercury is naturally present in the environment and is also released from anthropogenic activities (see also Figure 1.1). Inorganic mercury is converted to organic MeHg via microbial activity in the environment. MeHg is composed of a methyl group (CH_3-) bound to a mercury atom (Figure 1.4) and it has very high affinity for sulfur-containing anions, particularly the thiol (-SH) groups on the amino acid cysteine. When MeHg is ingested, it is almost totally absorbed from the gastrointestinal

tract and enters the blood stream to bind to hemoglobin. MeHg has a half-life in human blood of about 50 days (IPCS, 1990) and it is not readily eliminated, thus leading to accumulation in the body. It is mostly found complexed with free cysteine, as well as with cysteine-containing peptides and proteins. The methylmercuric-cysteinyl complex is recognized by amino acid transporting proteins in the body as methionine, another essential amino acid (Kerper *et al.*, 1992). Because of this mimicry, it is transported freely throughout the body including across the BBB and the placenta.

MeHg poses a risk to public health as it can affect the development of the brain of infants and can cause neurological changes in adults. It became clear that the developing nervous system is more vulnerable to MeHg than the adult nervous system

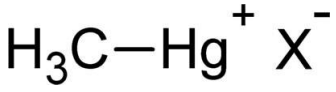

Figure 1.4 Schematic depiction of the MeHg molecule. Anions (X^-) that can readily combine with MeHg include chloride, hydroxide, and nitrate.

as during the outbreaks some mothers with no obvious symptoms of nervous system damage gave birth to infants with severe disabilities (Harada, 1995). There is also evidence of delayed neurotoxicity that appeared only with aging (Rice, 1996). Beside neuropathological damage, MeHg has also been found to affect the behavior of experimental animals (see (Burbacher *et al.*, 1990)). *In vitro*, it affects proliferation, migration and differentiation of neuronal stem cells (NSCs) (Burke *et al.*, 2006; Sass *et al.*, 2001; Tamm *et al.*, 2006). MeHg causes neurotoxicity by perturbation of intracellular calcium levels, induction of oxidative stress by producing more reactive oxygen species (ROS) or by reducing the cellular oxidative defense and also by interaction with sulfhydryl groups. Recently, Bose and colleagues provided novel evidence showing inheritance of programming effects induced by exposure to MeHg in NSCs (Bose *et al.*, 2012). This supports the idea that developmental exposure to low levels of MeHg may result in long term consequences predisposing to neurodevelopmental disorder and/or neurodegeneration. Hopefully, environmental catastrophes involving MeHg will not happen again because of the increased awareness and drastically limited and controlled industrial use. However, the current limited knowledge regarding the consequences of the prolonged exposure to environmentally relevant concentrations of MeHg gives reasons for concern.

1.3 EXPERIMENTAL MODELS AND METHODS IN NEUROTOXICOLOGY

Understanding the implications of exposure to neurotoxicants particularly during development is puzzling and is hampered by the complexity and heterogeneous structure of the nervous system. *In vitro* models offer unique opportunities to understand the molecular mechanisms of neurotoxicity. Whereas *in vivo* models offer a possibility to understand the effects of neurotoxicants in intact organisms that can be translated into changes in behavior.

1.3.1 *In vitro* methods and relevant endpoints

In vitro models are proven powerful methods for the investigation of cellular function perturbations induced by neurotoxic agents. Cultured cells originating from the nervous system have been in use for many years in neurotoxicological research and provide an important tool for mechanistic studies at molecular level. The reduced complexity as compared to *in vivo* facilitates the detection of changes in key cellular processes such as proliferation and differentiation. Therefore, *in vitro* experiments favor the distinction between cell-intrinsic and environmental mechanisms regulating development at cellular level (Qian *et al.*, 2000). At the same time, *in vitro* models do have some limitations, such as diminished metabolic capability; lack of complex interactions between cells; lack of coordinated expression of molecular and cellular events in a time- and region-dependent manner (Rice & Barone, 2000; Rodier, 1994; Rodier, 1995). It is important to be aware of these disadvantages and to take them into consideration when interpreting the results (Bal-Price *et al.*, 2008).

Both cell lines and primary cultures have been used as *in vitro* experimental models for neurotoxicity studies. By definition, cell lines are cultures that have been serially transplanted or subcultures from one culture vessel to another for a number of generations (Hertz *et al.*, 1985). Cell lines can be maintained in culture for an extended period of time given appropriate fresh medium and space. Immortalized cell lines have acquired the ability to proliferate indefinitely either through random mutation or deliberate modification, such as artificial expression of the telomerase gene. With each subsequent subculture, the cell population becomes more homogenous as the faster growing cells predominate. Cells with appropriate properties can be selected for specific studies. Examples of commonly used neuronal cell lines include the pheochromocytoma PC12 cells, and the neuroblastoma SH-SY5Y cells. Advantages in using cell lines include convenience and reproducibility, and a reduction in the use of animals. Cells harvested directly from the organism and maintained *in vitro* for periods exceeding 24 h are defined as primary cell cultures (Fedoroff, 1977). Primary cultures are typically a mixture of cell types. However, appropriate laboratory procedures can be used to select the desired cell type from the mixture. These can be made with or without an initial fractionation step to separate different cell types. Examples of primary cultures used for neurotoxicity studies are rat cerebellar granule cells, cortical and hippocampal neurons. Recently, embryonic neural stem cells (NSCs) of human and rodent origin have been introduced as *in vitro* models for neurodevelopmental toxicity studies (Moors *et al.*, 2009; Tofighi *et al.*, 2011). NSCs have the capacity of self-renewal and can generate all three major cell types of the CNS; neurons, astrocytes and oligodendrocytes (Doetsch, 2003; Temple, 2001; Zhang *et al.*, 2001). Importantly, the temporal sequence of cell lineage differentiation *in vivo* (first predominantly neurons, later predominantly glia) appears to be preserved in NSC primary cultures (Qian *et al.*, 2000). NSCs isolated from CNS have limited life span and either differentiate spontaneously or enter an irreversible growth arrest after a finite number of cell division (Caldwell *et al.*, 2001).

Cellular endpoints commonly used in neurotoxicological studies include cytotoxicity, proliferation, migration, differentiation, and cell death. Additional endpoints relevant

for neurotoxicity assays include neurite electrical activity, neurotransmitter release, and neurite outgrowth. The following section reviews only the endpoints considered in our studies.

1.3.1.1 Cell death

Cellular effects of toxicants may either disrupt normal cell function, which could lead to increased susceptibility to other forms of damage, ultimately inducing cell death. Death or survival of the cells in the presence of a chemical insult is often determined by the proliferative status, repair enzyme capacity, and the ability to activate proteins responsible to either promote or inhibit the cell death process (Orrenius *et al.*, 2011). Several types of cell death have been described *in vitro*, as well as *in vivo*, with apoptosis and necrosis being the most important.

Apoptosis is a programmed type of cell death and it is fundamental for both development and homeostasis in all tissues. Thus, superfluous, or damaged cells (infected, transformed) are normally removed by activation of an intrinsic program. Apoptosis is tightly regulated and ectopic activation can be catastrophic. Several factors are involved in the activation process during development such as cell lineage information, extracellular survival factors and hormones. In the CNS, neurons are produced in higher numbers than needed. The increase in the number of neuronal cells is followed by a wave of apoptosis to establish the appropriate final number of neurons (Oppenheim, 1991; Raff *et al.*, 1993). Cells undergoing apoptosis usually exhibit a characteristic morphology, including fragmentation of the cell into membrane-bound apoptotic bodies, nuclear and cytoplasmic condensation and endolytic cleavage of DNA into small oligonucleosomal fragments (Steller, 1995), generating high molecular weight fragments of 300 and 50 kbp, and low molecular weight fragments of 180 bp (Oberhammer *et al.*, 1993; Tomei *et al.*, 1993). The cells or fragments are then phagocytized by macrophages, and the content of the cells never reaches the intercellular space. Externalization of phosphatidylserine (PS) facilitates the recognition of dying cells by neighboring cells (Henson *et al.*, 2001). Two major apoptotic pathways have been described extensively. The extrinsic pathway is initiated by activation of plasma membrane death receptors, such as CD95, TNFR1 and TRAIL that belong to the tumor necrosis factor superfamily, by their specific ligands (Wajant, 2002). In the intrinsic pathway, death signals act directly or indirectly on the mitochondria, releasing pro-apoptotic proteins such as cytochrome *c* (cyt *c*) into the cytosol and start the apoptosis machinery (Orrenius *et al.*, 2011). The apoptotic process is mediated by gene induction, with the synthesis of signal molecules, and is completed with the activation of well-known proteases, such as caspases (Gorman *et al.*, 1998). Several mammalian caspases have been identified and a number of them and their regulators have been shown to be vital also during development (Cecconi *et al.*, 1998; Madden & Cotter, 2008). Initiator caspases are activated by cleavage, and after being activated they in turn cleave and activate execution caspases. Finally, they proceed to process key structural and nuclear proteins causing the disassembly and death of the cell.

It has long been known that Ca^{2+} signals are critical for cell function, and the loss of homeostatic control results in cell death. Mitochondria are necessary organelles for the cells to preserve cytosolic Ca^{2+} concentration, and have been recognized to actively participate in the compartmentalization of intracellular Ca^{2+} (Carafoli, 2002).

Interestingly, high level of Ca^{2+} in the mitochondrial matrix can trigger outer mitochondrial membrane (OMM) permeabilization and will result in mitochondrial swelling and membrane rupture. Some chemicals have the ability to trigger apoptosis via Ca^{2+}-mediated mitochondrial permeability transition (MPT) (Orrenius *et al.*, 2003). Disruption of intracellular Ca^{2+} homeostasis might mediate the apoptosis-inducing effects of some toxicants. Calpains, Ca^{2+}-dependent proteases that have been shown involved in the execution of cell death can be activated by different range of Ca^{2+} concentrations in cells (Orrenius *et al.*, 2003). For the activation of μ or m calpains, micromolar or millimolar concentrations are needed. Calpains can cleave a broad range of proteins such as cytoskeletal proteins and proteins associated with cell membranes, but also procaspase-3 and -9. The calpain specific inhibitor, calpastatin, can in turn be cleaved by activated caspases indicating a cross talk between caspases and calpains in the regulation of cell death (Orrenius *et al.*, 2003).

There is considerable evidence that additional proteases such as cathepsins (lysosomal proteases) participate in apoptosis by cleaving the pro-apoptotic Bcl-2 proteins, such as Bid, which leads to Bax activation. However, lysosomal enzymes may play a role rather in the amplification, than in the initiation of the apoptotic process (Oberle *et al.*, 2010). The cathepsins have also been implicated in apoptotic processes occurring in CNS in neurodegenerative diseases and following ischemia (Houseweart *et al.*, 2003; Lieuallen *et al.*, 2001; Tsuchiya *et al.*, 1999; Yamashima *et al.*, 1998). In addition, lysosomal rupture has been recognized as a feature of oxidative stress-induced cell damage (Zdolsek *et al.*, 1993), and the oxidant-induced lysosomal permeabilization appears to be mediated by an increase in intracellular free Ca^{2+} (Smolen *et al.*, 1986).

Necrosis is considered a passive and energy-independent form of cell death often caused by a serious injury, which compromises the integrity of the cell membrane. As a result of unrestrained water and ion influx, necrotic cells burst and release the cell contents into the extracellular space. In intact tissues, this triggers inflammation (Majno & Joris, 1995). However, growing evidence have open the insight that necrosis might be an active, regulated and controllable process (Vande Velde *et al.*, 2000). In fact, necrosis also has been observed to occur in parallel with, rather than as an alternative pathway to apoptosis,(see (Henriquez *et al.*, 2008)). Secondary necrosis is a special condition occurring in cell cultures, where phagocytic cells are lacking. In this process, apoptotic cells and their fragments lyse in a process similar to necrosis.

1.3.1.2 Cell proliferation

The development of CNS is governed by an exquisite balance between neural stem/progenitor cell proliferation and differentiation to form a proper cytoarchitecture (Cremisi *et al.*, 2003; Ohnuma & Harris, 2003). At early stages of embryonic development the cell have a surplus of intrinsic proliferative potential (Andreeff *et al.*, 2000), which is necessary to increase the pool of NSCs. Cell proliferation is a process that will increase the number of cells as a result of cell growth and repeated cell division (Jorgensen & Tyers, 2004). The cell cycle (Figure 1.5) can be divided into functional (S and M), and preparatory (G1 and G2) phases. An effective cell cycle involves an orderly and unidirectional transition from one phase to the next. Intrinsic regulatory pathways are responsible for the precise ordering by enforcing a series of

checkpoints which allow the progression of cell cycle only after the completion of critical steps (Dehay & Kennedy, 2007). The cell cycle transitions rely on separate positive and negative regulatory circuits, controlled by intrinsic and extrinsic signals. Since the length of S, G2 and M phases in mammalian cells is relatively invariant, the transitions between these phases are controlled predominantly by intrinsic regulatory pathways. Extrinsic regulatory pathways function in response to environmental conditions or in response to detected cell cycle defects (Andreeff *et al.*, 2000).

As CNS progressively develops, proliferation become restricted, some cells continue to proliferate, while others will lose their proliferative potential and start to differentiate (Agathocleous & Harris, 2009). The NSCs can undergo symmetric or asymmetric division. Symmetric cell division of NSCs occurs earlier in development and will increase the number of progenitor cells (Morrison & Kimble, 2006). Later, NSCs undergo asymmetric cell division to produce various types of neurons and glia (Figure 1.5). NSCs are known to change their characteristics during development (Alvarez-Buylla *et al.*, 2001; Kageyama *et al.*, 2005; Temple, 2001); neuroepithelial cells, the first form of NSCs will gradually change their morphology into radial glia (Gotz & Huttner, 2005). Radial glia undergo asymmetric cell division, by which each radial glial cell gives rise to an intermediate progenitor committed to neuronogenesis and one radial glial cell (Figure 1.5) (Malatesta *et al.*, 2000; Miyata *et al.*, 2001; Noctor *et al.*, 2001; Tamamaki *et al.*, 2001). The transition to gliogenesis involve a transient return to the symmetric division of progenitors (Yuahasi *et al.*, 2010). The mechanisms underlying these remarkable changes in progenitor behavior and fate during development remain elusive.

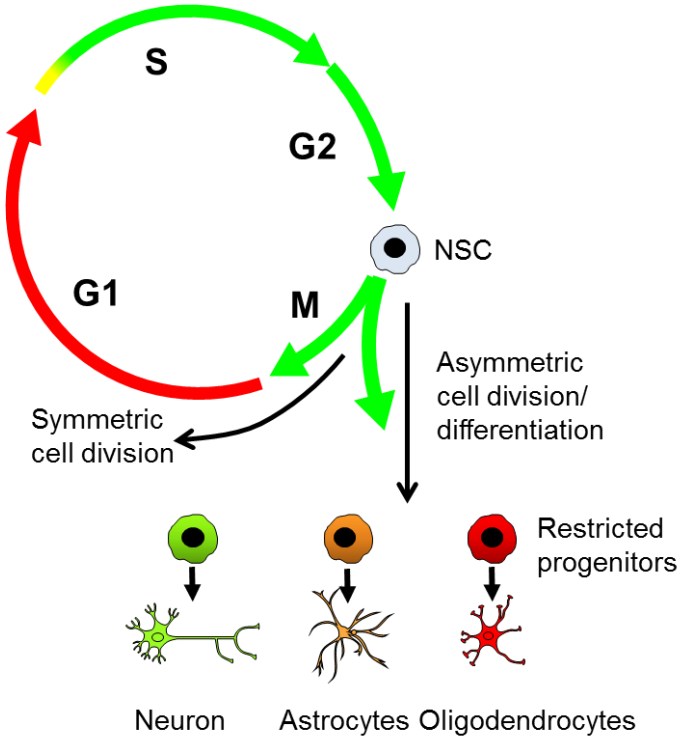

Figure 1.5 Typical cell cycle during cell proliferation. At early stage, neural stem cells divide symmetrically to increase stem cells pools. Later, when dividing asymmetrically or differentiating, the stem cells will exit the cell cycle to form developmentally restricted precursor cells, which finally differentiate into mature neurons, astrocytes or oligodendrocytes.

Exposure to toxicants may influence proliferation in particular cell populations. Pulse labeling for EdU (a nucleotide analog) incorporation, or the expression of markers such as Ki 67 or proliferating cell nuclear antigen (PCNA) can be used for assessing ongoing cell proliferation, while Trypan blue exclusion test can be used for estimating the total cell number and for identifying viable or apoptotic cells from necrotic cells.

1.3.1.3 Cell differentiation

Cell differentiation is a normal process by which stem cells/progenitor cells develops or matures to possess a more distinct form and function. This comprises a series of events involved in the development of a specialized cell having specific structural, functional, and biochemical properties. During neurodevelopment, a coordinated regulation of cell cycle exit and differentiation of neuronal precursors is essential for generation of appropriate number of neurons, neuronal subtypes and proper wiring of neuronal circuits (Politis *et al.*, 2008). Initiation of differentiation is apparently connected with the cell cycle control systems (Ohnuma & Harris, 2003) that instruct whether progenitor cells will maintain the proliferative capacity or become a committed precursors, which will subsequently exit the cell cycle and begin to differentiate (Figure 1.5). Cell cycle exit may be essential for differentiation, but is not sufficient, and additional signaling factors are needed (Tang *et al.*, 1999). In the developing brain, the NSCs are multipotent cells and generate a progressively restricted repertoire of cell types, in a precise sequence: first neurons, followed by oligodendrocytes and astrocytes (Bayer & Altman, 1991; Desai & McConnell, 2000; Temple & Qian, 1996).

In primary cell cultures, the proliferative capacity of NSCs can be maintained by supplying specific growth factors, such as basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). NSCs can be induced to differentiate into different cell lineage (*i.e.* neuron, oligodendrocytes and astrocytes) by adding different types of growth factors or cytokines to the culture medium. Thus, addition of platelet derived growth factor (PDGF), cilliary neurotrophic factor (CNTF), bone morphogenic proteins (BMPs), or the thyroid hormone T3, can direct the commitment depending on the developmental stage when the NSCs were derived (Gross *et al.*, 1996; Johe *et al.*, 1996; Li *et al.*, 1998; Panchision & McKay, 2002). In contrast, spontaneous differentiation can be induced by removing the mitogens (*e.g.* bFGF and EGF) (Johe *et al.*, 1996; Ostenfeld & Svendsen, 2004; Schwindt *et al.*, 2009).

Neurons are specialized, impulse conducting cells that are the functional units of the nervous system. Visualization of neurons *in vitro* is possible with different types of immunostainings such as neuron specific class III beta-tubulin (Tuj 1) and microtubule associated protein 2 (MAP 2). Oligodendrocytes are producing the myelin sheath of the axons, and are required for saltatory impulse conduction and for maintaining axonal integrity and long-term neuronal survival (Kassmann *et al.*, 2007). They can be visualized with immunostainings, such as against 2', 3'-Cyclic-nucleotide 3'-phosphodiesterase (CNPase). Astrocytes are star-shaped cells that play important role in supporting neuronal function and survival. Recently, astrocytes have been suggested to play an active role in neuronal communication via voltage-gated channels and

neurotransmitter receptors (Haydon & Carmignoto, 2006). Visualization of astrocytes can be achieved by immunostainings for glial fibrillary acidic protein (GFAP).

1.3.1.3.1 Signaling pathways

The acquisition of neural fate and neural patterning in NSCs is controlled by different signaling pathways, such as Notch, Wnts, Hedgehog, Shh (see (Guruharsha *et al.*, 2012)). Recently, peroxisome proliferator-activated receptors (PPARs) have also been shown to play a role in the differentiation of NSCs (Cimini *et al.*, 2007). Integration of, and crosstalk between different signaling pathways influence proliferation, differentiation, and cell death depending on the cellular context of signaling pathways activity (Guruharsha *et al.*, 2012; Louvi & Artavanis-Tsakonas, 2006). Here we review only the two signaling pathways that have been investigated in our studies: Notch and PPARs.

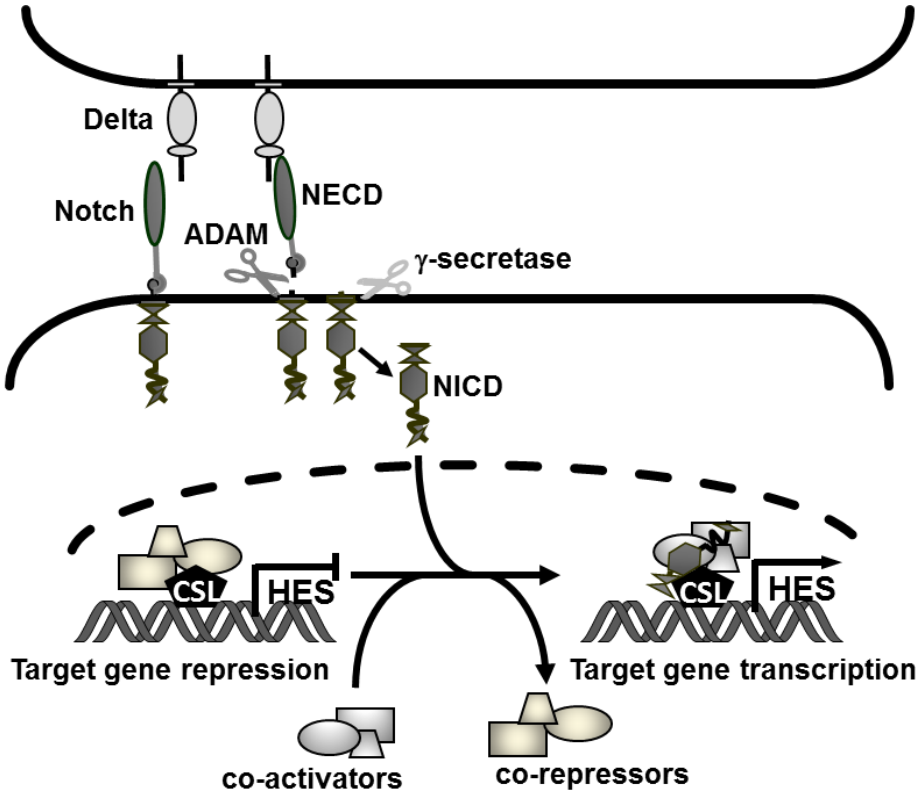

Figure 1.6 Simplified diagram of Notch signaling. Ligand binding to Notch receptors induces sequential receptor cleavage on the extracellular and intracellular sides of the membrane by ADAM and γ-secretase proteases. The Notch intracellular domain (NICD) translocate to the nucleus where it associates with the DNA-binding protein CSL and acts as a transcription regulator for neuronal differentiation pathways. In the absence of NICD, the CSL may associate with co-repressor to repress transcription of some target genes. Upon NICD binding, CSL will recruit co-activator to activate transcription.

Notch is well-known to play a vital role in regulating NSCs and neural development (Louvi & Artavanis-Tsakonas, 2006; Yoon & Gaiano, 2005) and recently it has been linked to stem cell fate and maintenance also in adult tissues (Bigas & Espinosa, 2012; Liu *et al.*, 2010). Multiple cell fate decisions are influenced by Notch, including

proliferation, differentiation, apoptosis, migration and angiogenesis. Thus, perturbations in Notch signaling are associated with several developmental abnormalities and cancer (Artavanis-Tsakonas *et al.*, 1999; Fortini, 2012; Gridley, 2003; Louvi & Artavanis-Tsakonas, 2012). In NSCs, the Notch signaling pathway is known to inhibit neuronal differentiation and promote proliferation during neurogenesis to maintain the self-renewable state of NSCs both *in vivo* and *in vitro (G*ao *et al.,* 2009*;* Zhou *et al.*, 2010*)*. Notch receptors are single-pass transmembrane heterodimers that are activated upon binding their membrane-bound ligands (Delta and Jagged) on the neighboring cells. Ligand binding results in cleavage of transmembrane domain mediated by γ-secretase that will lead to subsequent release of the notch intracellular domain (NICD) into the cytosol. Translocation of NICD to the nucleus will form a complex with the DNA-binding protein (C-promoter binding factor 1 (CBF-1), suppressor of hairless (Su (H)), lin-12 and glp-1 (Lag-1)) (CSL). The NICD–CSL complex in turn acts as a transcriptional activator and induces the expression of basic helix-loop-helix (bHLH) transcription factors, such as the hairy and enhancer of split (Hes) and others (Figure 1.6) (see (Bray & Bernard, 2010)). The activation of Hes1 and Hes5 induces NSCs to proliferate and represses the expression of neurogenic transcription factors (Nakamura *et al.*, 2000; Ohtsuka *et al.*, 2001). The latter, such as Math1/2, Ngn1/2, Mash1 and NeuroD, are required for promoting neurogenesis and inhibiting gliogenesis (Farah *et al.*, 2000; Nieto *et al.*, 2001). They also appear essential for maintaining the 'stemness' in nearby NSCs via Notch signaling (Kageyama *et al.*, 2005).

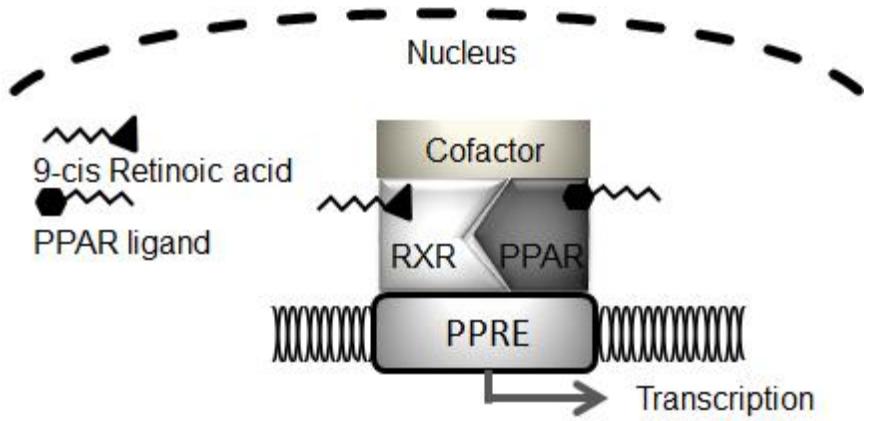

Figure 1.7 PPAR transcriptional regulatory complexes. PPARs activate the transcription of their target genes by forming heterodimer with RXRs. The function of PPAR/RXR heterodimer depends on its interactions with cofactor complexes (co-activators or co-repressors). Subsequent to activation by ligand, the PPAR/RXR heterodimer binds to specific DNA response elements, the PPREs, resulting in transcription of target genes.

The peroxisome proliferator-activated receptors (PPARs) are ligand-activated transcription factors that belong to the nuclear hormone receptor superfamily (Michalik *et al.*, 2006). There are three types of PPARs (PPARα, PPARβ/δ and PPARγ), encoded by different genes. PPARs activate the transcription of their target genes by forming heterodimer with its preferential binding partner, retinoid X receptors (RXRs) (Keller *et*

al., 1993; Wolfrum *et al.*, 2001). The function of PPAR/RXR heterodimer depends on its interactions with cofactor complexes (co-activators or co-repressors). The co-activators and co-repressors can either stimulate or inhibit receptor-mediated gene expression (Michalik *et al.*, 2006). Upon activation by ligand binding, PPAR/RXR heterodimer binds to specific DNA response elements called peroxisome proliferator hormone response elements (PPREs), located in the promoter regions of downstream target genes. This results in transcription regulation of PPARs target genes (Michalik *et al.*, 2004) (Figure 1.7). It should also be noted that Tudor and colleagues has shown that a high percentage of PPARs and RXR receptor association can occur *in vivo* even in the absence of ligands (Tudor *et al.*, 2007).

Recently, PPARs have been reported to be involved in NSCs acquisition of a different specific cell fate. All three PPAR isotypes are involved in the regulation of cell proliferation, death, and differentiation, with different roles and mechanisms depending on the specific isotype and ligand, and on the different status of the respective cells (IJpenberg *et al.*, 2004). Cimini and collaborators have demonstrated the presence of all PPARs isotypes in NSCs (Cimini *et al.*, 2007). All three isotypes have a nuclear localization in agreement with their function as transcription factors. The effects of PPAR on cellular proliferation and differentiation are potentially important to organs such as the CNS. PPARα has been shown to play a role in astrocytic differentiation (Cristiano *et al.*, 2005), while PPARβ has been demonstrated to be critical in neuronal maturation in cortical NSCs (Cimini *et al.*, 2005), and also in a human neuroblastoma cell line (Di Loreto *et al.*, 2007). PPARγ has been reported to be involved in oligodendrocyte differentiation (Roth *et al.*, 2003; Saluja *et al.*, 2001).

1.3.1.3.2 Spontaneous Ca^{2+} oscillations

Ca^{2+} is a widespread cation in the human body and plays a major role in different metabolic processes in all cell types. Transmembrane gradients of Ca^{2+} concentrations can generate fluctuations even by a relatively small increase in the cytosolic concentration. These fluctuations give rise to signals that convey vital information controlling cellular processes (see (Uhlen & Fritz, 2010) for review). A key feature of newly forming cortical networks during pre- and early postnatal development is spontaneous and synchronized neuronal activity (Katz & Shatz, 1996; Khazipov & Luhmann, 2006). This correlated network activity is believed to be essential for the generation of functional circuits in the developing nervous system (Spitzer, 2006). In both primate and rodent brains, early electrical and calcium network waves are observed pre- and postnatally *in vivo* and *in vitro* (Adelsberger *et al.*, 2005; Garaschuk *et al.*, 2000; Lamblin *et al.*, 1999). These early activity patterns, which are known to control several developmental processes, including neuronal differentiation, synaptogenesis and plasticity (Rakic & Komuro, 1995; Spitzer *et al.*, 2004) are critical for the correct development and maturation of cortical circuitries. Although spontaneous Ca^{2+} signals in undifferentiated cells may persist for many days, they become less frequent at early stages of neural precursor differentiation (Ciccolini *et al.*, 2003). Thus, spontaneous Ca^{2+} activity in NSCs can be used as a parameter to estimate the stage of differentiation in NSCs.

1.3.2 *In vivo* methods and relevant endpoints

In vitro studies may provide a deeper insight into the mechanisms of toxicity, but mechanistic studies become much more relevant when the endpoints are applicable in animal models and ultimately lead to explaining the findings in human populations. To gain a better understanding of the functional deficits induced by exposure to neurotoxicants, the specificity of the effects should be evaluated in terms of behavioral changes. The analysis of animal behavior often provides robust and reproducible outcomes to identify functional alterations induced by substances with neurotoxic potential (Gimenez-Llort *et al.*, 2001; Onishchenko *et al.*, 2007; Rossi *et al.*, 1997).

Several tests can be performed sequentially on the same animal at different ages, and biochemical and molecular analysis of nervous tissue may identify the molecular mechanisms underlying the changes in behavior. Animal models ranging from primates to fish have been employed for behavioral analysis. The behavioral testing evaluates basic (motor, sensory), as well as complex (learning and memory, social interactions) CNS functions. The selected behavioral endpoints should match the objectives of the study and possibly reveal the neurotoxic mechanisms involved (Macphail & Tilson, 1995). However, the extrapolation to predictable effects in human populations implies uncertainties related to variations between species and the assumption of test validity for human behavior.

1.3.2.1 Behavioral analysis

Here we reviewed only behavioral tests that are relevant to this thesis (see Figure 1.8 for an illustration of some of the equipment used). All measureable behaviors in rodents rely on motor function and typically involve complex neural events. Motor activity can be directly affected by a variety of factors influencing development, and it is therefore important to have it assessed among the first. Locomotor activity in home cage or novel environment can be used to assess the normal motor functions, as well as stereotypical behaviors. The rotarod is a rotating cylinder on which the mouse must walk continuously in order not to fall off, and it can be used for assessing motor coordination and balance (Carter *et al.*, 2001; Rustay *et al.*, 2003). Latency to fall is recorded as dependent variable, and mice with deficits in motor coordination or balance tend to fall earlier in the test session. Neuromuscular abnormalities can be detected with simple measures of muscle strength. Coordination and grip strength are required for a rodent to hold its body suspended. The latency to fall from the inverted wire lid can be quantified and used as a measure of the muscle strength (Shinzawa *et al.*, 2008).

More complex emotion-related behaviors in rodents, such as ‘anxiety-like behavior’ or ‘depression-related behavior’ can be measured using elevated plus maze and forced swim tests, respectively. Elevated plus maze is a test that builds on the conflict between the tendency of rodents to explore a novel environment and the choice of safe (dark, enclosed) vs. the potential exposure to predators (bright, open areas) (Lister, 1987). The forced swim test estimates the learnt helplessness or despair, as measured by the time the animal spends floating in a tall cylinder filled with water (Crowley *et al.*, 2004). Mice put in the water will generally struggle and actively seek an escape route. After

some time, the animal may stop swimming and float instead, apparently having given up the search. Longer time spent floating is interpreted as depression-like behavior.

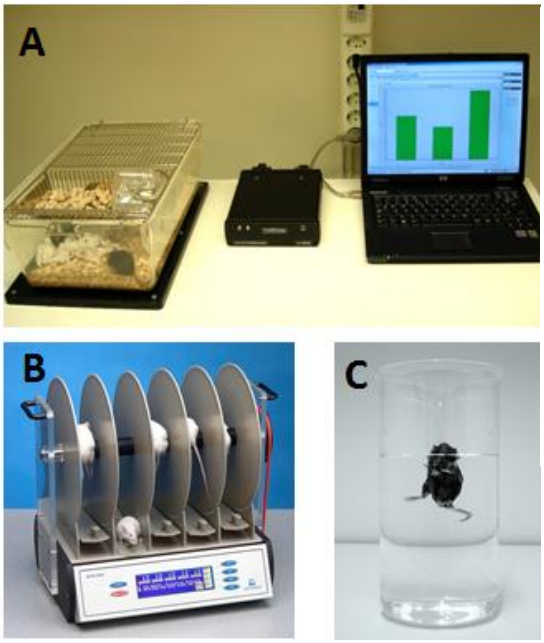

Figure 1.8 Illustrative images for behavioral testing used in this thesis. (**A**) TraffiCage ™ system for monitoring spontaneous locomotor activity in the homecage. (**B**) Rotarod system for testing motor coordination and balance. (**C**) Forced swim test to assess depression-like behavior. Images courtesy of Natalia Onishchenko (A and C) and Ugo Basile Srl (B).

Zebrafish models have become interesting in neurotoxicological screening, owing to a series of special characteristics. In addition to the readily available anatomic, physiologic, and thorough genetic characterization, the transparency during early developmental stages allows direct observations on organogenesis. Moreover, the rapid embryonic development, high fecundity, daily availability of high number of embryos and low cost husbandry, offer distinct cost, time and labor advantages compared to rodents. The earliest (4 days post fertilization (dpf)) behavioral response measurable in a high-throughput setting is the visual motor response (VMR), which consists of alterations in motor activity in response to light onset and offset (Ali *et al.*, 2012; Ellis *et al.*, 2012; Emran *et al.*, 2008). The organogenesis is completed by 6 dpf, and the larvae will have developed to a mature swimmer with functioning sensory and motor systems, thus allowing functional assessment to study different behaviors such as escape, goal oriented and optomotor response (Drapeau *et al.*, 2002; Guo, 2004; Kimmel *et al.*, 1995; Levin & Cerutti, 2009). Therefore, zebrafish emerge as an excellent alternative model for primary *in vivo* screening for toxicity of drugs, chemicals, and nanomaterials (Anderson & Ingham, 2003; Panula *et al.*, 2006; Xi *et al.*, 2010), as well as for neurobehavioral and pharmacological studies (Chakraborty *et al.*, 2009; Eddins *et al.*, 2010; Irons *et al.*, 2010; Rihel *et al.*, 2010; Steenbergen *et al.*, 2011).

2 AIMS

The studies included in this thesis aimed at investigating:

1. the mechanisms of MeHg, PCB 153 (NDL), and PCB 126 (DL) neurotoxicity, as well the possible synergistic/antagonistic effects of combined exposure, with special focus on cell death pathways

2. the effects of low concentrations of NDL-PCBs, MeHg and PFOS on NSCs differentiation

3. the potential toxic effects of prenatal exposure to low levels of PFOS or PFOA during neurodevelopment, taking into account possible sex-related differences.

3 MATERIALS AND METHODS

3.1 *IN VITRO* MODELS AND METHODS

3.1.1 Cell culture procedures and exposure

3.1.1.1 Cell cultures

In *Paper I*, we used the mouse hippocampal HT22 cells, a subclone derived from the HT-4 cells and immortalized with a temperature-sensitive SV40 antigen, which express neuronal properties (Lendahl & McKay, 1990; Morimoto & Koshland, 1990). HT22 cells were seeded at a density of 3 000 cells/cm^2, in CO_2-independent medium (LifeTechnologies, Gibco BRL) supplemented with 10% FCS, 4 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. The cell culture flasks were closed airtight and kept in 37^{0}C with proper humidity for 24 h before exposure to the toxicants.

In *Papers II and III*, we used primary cultures of NSCs obtained from embryonic cortices dissected in Hanks' Balanced Salt Solution (HBSS) (Life Technologies) from timed-pregnant Sprague–Dawley rats (Harlan Laboratories, The Netherlands) at embryonic day (E15) (the day of copulatory plug was defined as E0). The tissue was gently dispersed mechanically, and the meninges and larger cell clumps were allowed to sediment for 10 min. The cells were plated at a density of 40 000/cm^2 on dishes pre-coated with poly-L-ornithine and fibronectin (both from Sigma). Cells were maintained in enriched N2 medium (Bottenstein & Sato, 1979) with 10 ng/ml basic fibroblast growth factor (bFGF) (R&D Systems, Minneapolis, MN, USA) added every day and the medium changed every other day to keep cells in an undifferentiated and proliferative state. When still subconfluent, the cells were passaged after detaching by incubation with HBSS and subsequent scraping. Afterwards, the cells were mixed in N2 medium, counted, and plated at low density (500 cells/cm^2) on coverslips coated with poly-L-ornithine and fibronectin, and grown in the presence of bFGF. Two days after the passaging, the medium was changed without adding bFGF to promote

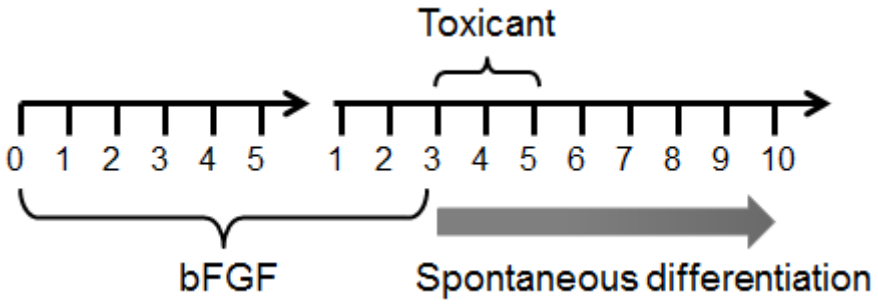

Figure 3.1 A scheme of experimental model. After dissection of the cortices and cell plating, NSCs were maintained undifferentiated for 5 days by adding bFGF. Then the cells were passaged and grown in the presence of bFGF for an additional 48 h. Then bFGF was withdrawn and the cells were exposed to toxicants. After 48 h exposure, the medium was changed and the cells were allowed to continue the spontaneous differentiation for an additional 5 days before being assayed.

spontaneous differentiation for the following 7 days. The cells were simultaneously exposed to toxicants directly in the culture medium. After 48 h exposure to the toxicants, the medium was changed every second day (Figure 3.1). Analysis of the NSCs proliferation and spontaneous differentiation was performed 7 days after growth factor withdrawal.

3.1.1.2 Exposure to chemicals

In *Paper I*, HT22 cells were exposed to 0.5-4 µM MeHg (stock concentration 0.5 mM), 50-200 µM PCB 153 (stock concentration 50 mM), and 12.5-50 µM PCB 126 (stock concentration 12.5 mM) either alone or in combinations for up to 24 h.

In *Paper II*, NSCs were exposed to 25-100 nM PCB 153 or 25-100 nM PCB 180 (stock concentration 100 µM) or 5 nM MeHg (stock concentration 5 µM) for 48 h, upon withdrawal of bFGF. To exclude possible contamination by Dioxin-like compounds, both PCB 153 and 180 (Neosync Inc., USA), were purified from polychlorinated dibenzodioxins/polychlorinated dibenzofurans (PCDD/Fs) and Dioxin-like-PCBs, by applying the PCBs dissolved in n-hexane on an active carbon column and collecting them after elution with n-hexane. The purified PCBs (impurity levels <pg/g) were dissolved in purity-checked DMSO (Danielsson *et al.*, 2008).

In *Paper III*, NSCs were exposed to 12.5-100 nM PFOS (stock concentration 12.5-100 µM) for 48 h.

PCBs or PFOS were diluted in DMSO and control cells were exposed to the same amount of DMSO (0.1–0.4%) in *Paper I* or (0.1%) in *Papers II and III.* MeHg was diluted in water.

3.1.1.2.1 Combined exposure

In *Paper I* we exposed HT22 cells to a combination of Me Hg, PCB 126, and PCB 153 at non-cytotoxic concentrations (0.5 µM MeHg, 50 µM PCB 153, and 12.5 µM PCB 126), or sub-cytotoxic concentrations (1 and 2 µM MeHg, 100 µM PCB 153, and 25 µM PCB 126).

In *Paper II* we exposed NSCs to a combination of 5 nM MeHg and 100 nM PCB 153 or PCB 180.

3.1.1.3 Treatment with proteases inhibitors and antioxidants

In *Paper I*, the caspase inhibitor z-VAD-fmk (20 µM), the cysteine protease inhibitor E64d (25 µM), the calpain specific inhibitor PD150606 (100 µM), the cathepsin D inhibitor Pepstatin (100 µM), the antioxidant MnTBAP (100 µM), and NAC (10 mM) were added 30 min prior exposure to the toxicants and left in the culture for the entire exposure period.

In *Paper II*, cells undergoing spontaneous differentiation were exposed daily to γ-secretase inhibitor, DAPT (2.5 mM) to block the cleavage of Notch receptors.

In *Paper III*, cells were simultaneously exposed to PFOS or the PPARγ agonist rosiglitazone (RGZ) (3 µM), or pre-incubated with the PPARγ antagonist, GW9662 (5 µM) for 60 minutes before exposure to PFOS upon withdrawal of bFGF.

3.1.2 Cell morphology and viability

3.1.2.1 Trypan blue

At the end of exposure, cells were washed with HBSS or Hank's solution, then trypsinized (*Paper I*) or scraped (*Papers II and III*), and the single cell suspension was mixed with an equal amount of 0.4% Trypan blue solution (membrane impermeable dye). Cells were scored at the phase contrast microscope using an improved Neubauer counting chamber. Cells with damaged cell membrane stained blue (necrotic) while cells with intact membrane (healthy or apoptotic cells) remained unstained. Total cell number was then calculated as number of cells which are stained plus unstained.

3.1.2.2 Vital triple staining

In *Paper I*, we performed vital triple stainings on cells grown on coverslips to distinguish apoptotic cells from necrotic. The cells were incubated with a cocktail solution containing Hoechst 33342 (1 µg/ml), Propidium Iodide (PI) (1 µg/ml) and Annexin V-FITC (0.5 µg/ml) in a buffer solution containing 10 mM HEPES/NaOH (pH 7.4), 140 mM NaCl, and 2.5 mM $CaCl_2$. Apoptotic cells were identified by the condensed chromatin (stained by Hoechst 33342) and exposed phosphatidylserine (PS) (stained by Annexin V) on the surface of cell membrane, while necrotic cells were recognized by positive PI-staining due to damaged cell membranes. The cells were analyzed with an Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan).

3.1.2.3 Nuclear staining

In order to detect apoptosis for *Papers II and III*, NSCs were grown on poly-L-ornithine/fibronectin coated coverslips and fixed in 4% paraformaldehyde (PF) for 1 h at 4°C. After washing with phosphate-buffered saline (PBS), cells were stained with Hoechst 33342 (1 µg/ml) for 5 minutes at room temperature (RT), then rinsed with PBS. After mounting, cells were analyzed with a fluorescent microscope and at least 100 nuclei were counted in 5 non-overlapping fields per coverslip. Apoptotic cells were identified by the condensed chromatin.

3.1.3 Cell proliferation

In *Papers II and III* we assessed NSCs proliferation using EdU, a thymidine analogue, containing a terminal alkyne group that readily gets incorporated into cellular DNA during S-phase. The terminal alkyne group is then detected through its reaction with fluorescent azides. Cells were spontaneously differentiated and exposed to the toxicants as described above and 10 µM EdU was added to the culture media 1 h prior to fixation. Cells were then rinsed with PBS and incubated with freshly prepared mix of 100 mM Tris (pH 8.5), 1 mM $CuSO_4$, 10 µM Alexa 488-azide, and 100 mM ascorbic

acid for 30 min. After staining, the cells were washed with PBS and counterstained with Hoechst 33342 before mounting. For quantification, at least 100 nuclei were counted in 5 non-overlapping fields per coverslip.

3.1.4 Cell cycle analysis

In *Paper II, c*ells were transfected using the fluorescence ubiquitination cell cycle indicator (FUCCI) plasmids (a kind gift from Dr. Atsushi Miyawaki), to analyze cell cycle progression following treatments (Sakaue-Sawano *et al.*, 2008). The two plasmids encoding green and red fluorescent proteins fused with E3 ligase substrates indicate whether live cells are in G1 (non-proliferating) or S/G2/M (proliferating) phase. Transfection was performed using Lipofectamine 2000 (Invitrogen) according to manufacturer's protocol and the efficiency was > 10%. The number of proliferating (green) and non-proliferating (red) nuclei was assessed 48 hours after transfection.

3.1.5 Lysosomal integrity

In *Paper I, d*amage of the lysosomes was measured in non-fixed cells using the lysosomotropic weak base acridine orange (AO). At the low pH of the lysosomes, AO becomes charged (AOH^+) and consequently membrane impermeable, thus it is retained into these organelles. The AO concentrated in the lysosomes has a monochromatic red fluorescence when excited with green light. The amount of the red fluorescence per cell is indicative of lysosomes with high AO-concentration and intact proton gradients. HT22 were grown on coverslips and exposed to the toxic agents, then incubated with AO-medium (5 µg/ml AO in complete medium pre-equilibrated at 37^0C for 15 min) and washed with fresh medium before measurements. The analysis was performed by assessing the variance in lysosomal granularity between control and treated samples.

3.1.6 Mitochondrial functions

3.1.6.1 Analysis of mitochondrial Ca^{2+} uptake

Paper I: The rate of mitochondrial Ca^{2+} uptake was investigated in cells permeabilized with 0.005% digitonin using a Ca^{2+} sensitive electrode (Orion Research, Beverly, MA, USA), as previously described (Ahlbom *et al.*, 2000). In our experimental conditions, addition of Ca^{2+} to permeabilized cells resulted in a rapid elevation of Ca^{2+} level in the buffer followed by a time-dependent decrease due to Ca^{2+} uptake by the mitochondria. The sequential additions of Ca^{2+}-induced mitochondrial permeability transition followed by Ca^{2+} release. The Ca^{2+} capacity, defined as the threshold of Ca^{2+} necessary for mitochondrial permeability transition induction, was expressed as nmol Ca^{2+} (in the buffer)/10^6 cells.

3.1.6.2 Mitochondrial membrane potential (ΔΨ)

Paper I: Tetramethylrhodamine ethyl ester (TMRE) is a dye that partitions to the negatively charged mitochondrial matrix according to the Nernst equation and acts as a voltage sensitive probe. Decreases in ΔΨ are paralleled by a reduction of the fluorescence emitted by TMRE (Daré *et al.*, 2001; Ehrenberg *et al.*, 1988). Cells grown

on coverslips were incubated with 5 nM TMRE in PBS for 30 min at RT and co-stained with Hoechst (1 µg/ml) for 5 min. The mitochondria and nuclei were analyzed by confocal microscopy (Zeiss LSM 510 meta).

3.1.6.3 Intracellular ATP determination

Paper I: Cellular ATP concentrations were determined in a luminometric assay using the ATP dependency of the light emitting luciferase-catalyzed oxidation of luciferin (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's protocol. Briefly, cells (5.0×10^4) were resuspended in 50 µl PBS and 450 µl of boiling lysing buffer (100 mM Tris, 4 mM EDTA, pH 7.75) were added. Samples were incubated for another 2 min at 100^0C and 100 µl were taken out to a 96-well plate. Prior to measurement, 100 µl of luciferase was added to each well and the plate was analyzed in a luminometer (Berthold, R-Biopharm AG, Germany).

3.1.7 Caspases activity

Paper I: We measured caspase activity by using a fluorogenic assay that evaluates the activity of class II caspases (caspase 2, 3, and 7), as previously described (Gorman *et al.*, 2000). Substrate cleavage leading to the release of free 4-methylcoumaryl- 7-amide (excitation 355 nm, emission 460 nm) was monitored at 37^0C using a Fluoroscan II (Labsystem AB, Stockholm, Sweden). Fluorescence units were converted to pmoles of 4-methyl-coumaryl-7-amide release using a standard curve generated with 4-methyl-coumaryl- 7-amide and subsequently related to protein content.

3.1.8 Spontaneous Ca^{2+} oscillations

Papers II and III: Cells were incubated (30 min at 37^0C in 5% CO_2) in cell culture medium containing 5 µM Fluo-3/AM (Molecular Probes) together with 0.1% Pluronic F-127 (Molecular Probes). After rinsing the cells, KREBS-Ringer's solution was added (119 mM NaCl, 2.5 mm KCl, 1 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.3 mM $MgCl_2$, 20 mM HEPES, 11 mM D-glucose). Petri dishes were transferred to a Zeiss LSM 510 META scanning laser confocal microscope equipped with a ×20/1.0 dipping lens (Zeiss) and images were acquired at 0.2 Hz. To determine the number of active cells, a spontaneously active cell was defined as a cell that displayed at least two well defined spontaneous Ca^{2+} peaks in 10 minutes, where each peak value was an increase in Ca^{2+} of more than 15% compared with the baseline. At the end of the experiment, 100 µM glutamate was bath-applied. Spectral analysis of Ca^{2+} oscillations and response to glutamate was performed with MATLAB software as described previously (Uhlen, 2004).

3.1.9 Immunocytochemistry

Paper I, II and III: Cells grown on coverslips were fixed in 4% PF (Sigma Aldrich, Sweden) for 1 h at 4^0C followed by washing in PBS. Primary antibodies (see Table 1) were diluted in PBS containing 0.3% Triton X-100 and 0.5% bovine serum albumin (Boehringer Mannheim). The fixed cells were incubated overnight in a humid chamber at 4^0C. Cells were then rinsed with PBS and incubated with appropriate secondary

FITC- or Texas-red-conjugated antibodies for 1 h at RT (1:200; Jackson ImmunoResearch, West Grove, PA, USA or Alexa, Molecular Probes, Invitrogen). Cell nuclei were counterstained with Hoechst 33342 (1 µg/ml). After rinsing with PBS, coverslips were mounted with glycerol-PBS containing 0.1% phenylenediamine (*Paper I*) or Vectashield® mounting medium (Vector Laboratories, Inc, USA) (*Papers II and III*). Images were captured using an Olympus BX60 fluorescence microscope (Olympus, Tokyo, Japan) equipped with a Hamamatsu digital camera (C4742-95-10sc, Hamamatsu Photonics Norden AB, Solna, Sweden). The mitochondria and nuclei were analyzed by confocal microscopy (Zeiss, LSM 510 Meta) (*Paper I*) otherwise cells were examined using fluorescence microscope (Nikon Eclipse Ti-S) and images were captured using Nikon camera (Nikon Digital Sight DS-Qi1MC) (*Papers II and III*). Image analysis was performed using Volocity Demo (Perkin Elmer) software where at least 100 nuclei were counted in 5 different fields per coverslip.

Table 1. Primary antibodies used for immunocytochemical staining

Paper	Antigen	Raised in	Dilution	Source
I	cyt *c*	Mouse	1:100	BD PharMingen
II	Nestin	Mouse	1:200	Chemicon
III	Nestin	Mouse	1:200	Milipore
II	GFAP	Rabbit	1:800	Dakocytomation
III	GFAP	Rabbit	1:800	Sigma
II	Tuj1	Mouse	1:400	Convance
III	Tuj1	Mouse	1:500	Convance
III	CNPase	Mouse	1:500	Sigma

3.1.9.1 Morphological analysis

In *Paper III*, Tuj1 or CNPase-positive cells were examined using fluorescence microscope (Nikon Eclipse Ti-S) and images were captured using Nikon camera (Nikon Digital Sight DS-Qi1MC). Image analysis was done using NIS Elements BR. Cells with processes properly isolated from other cells where the neurite ending could be clearly determined were used for measurement of neurite outgrowth (Figure 3.2 A). The length of the longest neurite was recorded. Measurement of CNPase positive cells

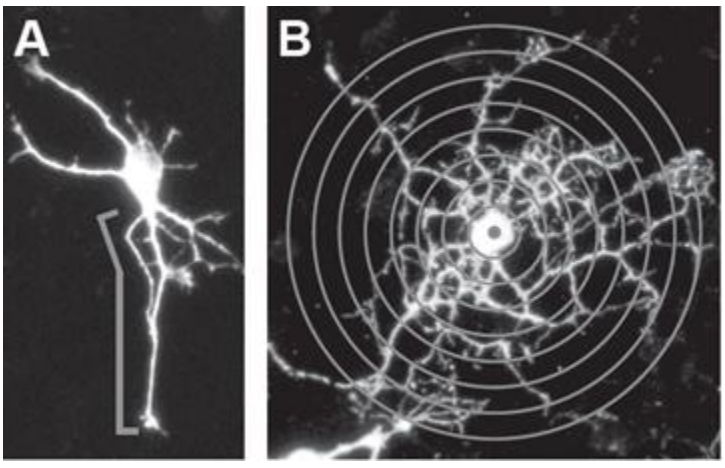

Figure 3.2 Measurement of neurite outgrowth (**A**) and measurement of arborization process of oligodendrocytes (**B**)

was done according to (Fernandez *et al.*, 2004) with some modifications. Briefly, to measure the arborization distance of CNPase positive cells, concentric circles with increasing diameter (16.5, 33.0, 49.5, 66.0, 82.5, 99.0, 115.5, 132.0 µm) were drawn from the nucleus (Figure 3.2 B). Every circle was then categorized into category 1 to 7. The nuclei of the cells were put in the smallest circle in the center and the furthest arborization of the cell was scored. The number of cells belonging to the different categories was counted. Measurement of neurite outgrowth or arborization process was made in 100 distinct neurite-bearing Tuj1-positive cells or CNPase-positive cells selected according to the criteria above.

3.1.10 Immunoblotting

Papers I and II: Cells were harvested with trypsin or scraping, centrifuged and washed with PBS. Then, cells were sonicated in a solution containing 1 mM Pefablock (Boehringer Mannheim, Bromma, Sweden), 10 mM EDTA and 2 mM DTT in PBS. Protein content was determined using NanoDrop 1000 spectrophotometer (Thermo Scientific Wilmington, DE, USA). After adding sample buffer (0.4% sodium dodecyl sulfate (SDS), 4% glycerol, 1% β-mercaptoethanol, 12.5 mM Tris-HCl, pH 6.8), 50-100 µg total protein was boiled for 5 min and subjected to 10-12% SDS-polyacrylamide gel electrophoresis followed by electroblotting to nitrocellulose membrane.

In *Paper I*, membranes were incubated overnight at 4^0C with a mouse anti-spectrin monoclonal primary antibody for 1 h in RT (dilution 1:1,000, Chemicon, CA, USA) and a horseradish peroxidase conjugated goat anti mouse secondary antibody (dilution 1: 20,000, Pierce Rockford, IL USA).

In *Paper II*, membranes were incubated with a polyclonal primary antibody against total Notch 1 (C-20, 1:1000; Santa Cruz) and a horseradish peroxidase conjugated secondary antibodies (1: 10 000, Pierce Rockford, IL USA) for 1 h in RT.

Equal protein loading was verified with rabbit anti-GAPDH (1:3000, Nordic Biosite, Täby, Sweden). Then membranes were rinsed again and developed with ECL reagents (Amersham,GE Healthcare, Buckinghamshire, UK) and exposed to X-ray autoradiography films (FujiFilm, Japan).

3.1.11 Quantitative real-time PCR (q-PCR)

Papers II and III: Total RNA was isolated using the RNeasy Mini Kit (Qiagen, VWR, Stockholm, Sweden) (*Paper II*) or peqGold Total RNA kit (peQ Lab GmbH, Erlangen, Germany) (*Paper III*). Integrity and concentration of extracted RNA was measured by NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA). cDNA was prepared using 1 µg total RNA and 0.5 µg of Oligo-dT primer following the instructions of Superscript II first strand cDNA synthesis kit (Invitrogen Inc., Carlsbad, CA, USA). Amplification reactions were performed with 1 µl cDNA, SYBR Green Mix (Applied Biosystems) and 0.2 µM of forward and reversed primers. The primers used are listed in Table 2. The reaction volume was adjusted to 25 µl with DEPC water. Negative control reactions contained water instead of cDNA template. q-PCR was

performed using an ABI Prism 7500 Sequence Detection System with SDS version 2.1 software (Applied Biosystems). The PCR cycle conditions were 50^0C for 2 min, 95^0C for 10 min, 95^0C for 15 s and 60^0C for 1 min (40 cycles). To evaluate the amplification of a specific sample, final melting curve (from 60^0C up to 95^0C) was added under continuous fluorescence measurements. For quantification of the relative mRNA levels, the following equation provided by the Perkin-Elmer Instruction Manual of 1997 was utilized: relative change $=2^{-\Delta\Delta CT}$, where C_T is the point (cycle) at which the amplification plot crosses the threshold and $\Delta\Delta C_T = (\Delta C_{T\ exposed} - \Delta C_{T\ controls})$, where the relative amount of target mRNA (ΔC_T) has been normalized to housekeeping gene such as CXXC1.

Table 2. List of primers used for qPCR analysis

Paper	Functional cluster	Gene symbol		Primers
II & III	Rat Notch signalling	Hes5	LP RP	tagtcctggtgcaggctctt atgctcagtcccaaggagaa
		Math1	LP RP	tggacagcttcttgtcgttg ttccagcaaacaggtgaatg
III	Rat PPAR signaling	PPARα	LP RP	cattctgcgacatcatggaa aatcccctcctgcaacttct
		PPARβ	LP RP	tcaacaaagacggactgctg gaaaagggccagatcactgt
		PPARγ	LP RP	ccctggcaaagcatttgtat actggcacccttgaaaaatg
		UCP 2	LP RP	gccacttcacttctgccttc gaaggcatgaacccttgta
		UCP 3	LP RP	ccggtggatgtggtaaagac ttaaggccctcttcagttgc
	Mouse PPAR signaling	PPARα	LP RP	tcacaagtgcctttctgtcg tgctttcagttttgctttctca
		PPARβ	LP RP	gaacagccacaggaggagc gaggaaggggaggaattctg
		PPARγ	LP RP	ggaagccctttggtgacttt acgtgctctgtgacgatctg
		UCP 2	LP RP	actgtgcccttaccatgctc catggagaggctcagaaagg
		UCP 3	LP RP	ccggtggatgtggtaaagac ttaaggccctcttcagttgc
II & III	Housekeeping genes	Rat CXXC1	LP RP	atcgtgttggatggtttggt gaggtctgtgggtgtccact
III		Mouse CXXC1	LP RP	cagacgtcttttgggtcca agacctcatcagctggcac

3.2 ANIMALS EXPOSURE AND *IN VIVO* METHODS

3.2.1 Animal exposures

Papers III and IV: All animal experiments were performed in accordance with the rules of the Swedish animal protection legislation and were approved by the local Animal Ethics Committee (Stockholms Norra Djurförsöksetiska Nämnd). C57BL/6/Bkl (Scanbur BK, Sweden) female mice were mated with males overnight and the next morning was considered gestation day (GD) 1 if a vaginal plug was observed. Exposures to PFOS or PFOA were performed as two separate experiments with their own control group (set 1: PFOS + Control1; set 2: PFOA + Control2). Pregnant dams received PFOS (n = 6) as heptadecafluorooctanesulfonic acid potassium salt (purity ≥ 98%, Sigma-Aldrich) or PFOA (n = 6) (purity 96%, Sigma-Aldrich) at the dose of 0.3 mg/kg/day via food from GD1 throughout pregnancy. PFCs were dissolved in 95% ethanol at the concentration of 1 mg/µl, and the solutions were applied on palatable food in a volume adjusted according to the individual body weight to reach the exposure dose of 0.3 mg/kg. The food bits were kept on the bench for 2 h to let ethanol evaporate and then placed in the cages. Control females (n = 10 in total) received similar bits of the palatable food with the vehicle applied and then evaporated.

In *Paper III*, the offspring from mothers exposed to 0.3 mg/kg/day PFOS and from vehicle group were sacrificed on the postnatal day (PND) 1. The cortical regions of the brains were cut out, and then the samples were disrupted and homogenized using a pestle in peqGold lyses buffer followed by RNA extraction with the peqGold columns.

In *Paper IV*, offspring were separated from mothers on PND 21 and were injected subcutaneously with sterile microtransponders (ID-100A, Trovan, Ltd., UK) under inhalation anesthesia (4% isoflurane in breathing air). Each transponder had an individual number that was used for animal identification. One or two offspring from the same litter were randomly selected for inclusion in the experimental groups. The mice were housed in groups of 3–4 animals per cage and the social groups were preserved throughout the experiments. Control and PFOS-exposed groups of both sexes consisted of 8 animals. PFOA-exposed groups included 6 males and 10 females, their respective control groups consisted of 8 male and 10 female mice. All animals were kept under standard laboratory conditions (21^0C, 12 h light–dark cycle with a light phase between 6.00 and 18.00) with free access to food and water.

3.2.2 PFOS and PFOA concentration in tissues

In *Paper IV*, tissue samples (whole brain and liver) (n = 4, one pup per litter) were collected from pups at birth. The concentration of PFOS and PFOA in the samples was measured with HPLC–MS by adapting the method previously described by (Maestri *et al.*, 2006).

3.2.3 Behavioral assessments

In *Paper IV*, tests for locomotor and circadian activity were performed at the age of 5–8 weeks. Afterwards, animals were tested for emotion-related behavior in elevated plus

maze and forced swim tests. Tests for muscle strength and motor coordination were performed in animals 3- to 4-month old.

3.2.3.1 Locomotor activity

The locomotor activity test was performed in cages made of transparent Plexiglas (42.5 x26.6x18.5 cm) with sawdust bedding covering the floor of the cage. Mice were individually placed in the new cages; behavior was video recorded and the walked distance was measured by using the automated video tracking system TopScan^{TM} (Clever Systems Inc., Reston, VA, USA). Distance travelled was registered in 5 min intervals over 30 min and used for statistical analysis.

3.2.3.2 Elevated plus maze test

A maze with a shape of a plus sign formed by two open arms (40 x 10 cm), two enclosed arms (40 x 10 cm) and a central platform (10 x 10 cm) was used. The apparatus, made in gray plastic, was placed 50 cm above the floor. Animals were released on the central platform facing one of the open arms, and allowed to explore the maze for 5 min. After the end of each test, the arena was carefully cleaned with 70% ethanol. Data were collected by using a TSE video tracking system (TSE Systems, Bad Homburg, Germany). Number of entries and time spent in the open arms as well as preference for visits to open or closed arms were used as parameters of anxiety-like behavior.

3.2.3.3 Forced swim test

Animals were individually placed in a glass cylinder (24 cm height, 12 cm diameter) filled with water (27^0C) up to a height of 16 cm for 10 min (pre-test) and 6 min (test; 24 h after the pre-test session) respectively. The sessions were video recorded and analysed offline for total duration of immobility. Immobility was defined as floating passively in the water at least 2 s or longer, without any movements or just small ones necessary to keep the head above the water surface. The inactive state (immobility) is considered to be a measure of depression-like behaviour and is potentiated by repeated testing.

3.2.3.4 Muscle strength in the hanging wire test

Mice were individually placed on the top of a wire cage lid and when it griped the wires, the lid was turned upside down. The lid was held upside down approximately 20 cm above the cage floor covered with sawdust bedding. Latency to fall off the lid was measured with 60 s cut-off time. The test was repeated 3 times with 30 min intervals. The average value over three trials was used for statistical analysis.

3.2.3.5 Accelerating rotarod test

Motor coordination in the exposed and control mice were evaluated using a rotarod (LE 8200, Letica Scientific Instruments, Barcelona, Spain). The test trials on the accelerating rod were performed after three training sessions (habituation with the

stationary and rotating rod, 4 rpm). The speed of the rotarod accelerated from 4 to 40 rpm over a 5-min period. Mice were placed on the rotating drum, and the time they remained on the rotarod was registered automatically. Mice were given four consecutive test trials with a maximum trial time of 300 s and 30 min between trial-rest intervals.

3.2.3.6 Circadian activity in the home cage

The mice were moved to the experimental room and placed in new cages preserving the social groups immediately before starting the activity recording. The cages were placed on TraffiCage^{TM} platforms (NewBehavior, Zurich, Switzerland). The platforms consisted of a plastic base (42.5 x 27 x 1.7 cm) with 5 embedded circular antennas (9 cm in diameter), which detect the presence of the injected transponders. When a mouse crossed from one antenna to another, the movement was recorded as crossing and used as activity count. The experiment started at 10 a.m. and the activity of the mice was monitored for 48 consecutive hours. The animals were kept under the same conditions (temperature, light dark cycle, food and water access) as described above throughout the duration of the experiment. The recordings were divided as follows: the first 3 h were analyzed separately as adaptation to a novel environment. Unlike tests of locomotor activity, animals evaluated for circadian activity remained in their original social group. Typically, the novelty-induced hyperactivity drops to baseline level within 3 h after putting the animals in a new cage. The remainder was considered normal homecage locomotor activity and was further divided into light and dark phases, according to the intensity of the light provided in the experimental room. In addition, we analyzed the resting time as the total duration of inactive periods. An ‘‘inactive period’’ was defined as a lag longer than 10 min between two consecutive crossings based on two observations: (1) close visual observation of the behavior in the homecage not detecting any activity (eating, drinking, grooming) in the territory of one single antenna that would last longer than 10 min; (2) the analysis of lags between crossings showed low incidence of lags longer than 7 min, but shorter than 12 min. Thus, by setting the threshold at 10 min, we were able to consistently distinguish long (inactive periods) from short lags (inherent to normal activity in the homecage), and assumed that lags longer than 10 min were indicative for resting. Because of the trailing effect of novelty-induced hyperactivity, we used only the second light-dark cycle for analysis of resting time.

3.3 STATISTICAL ANALYSES

Papers I, II and III: All experiments were performed in triplicates and repeated at least three times. Data were presented as mean ± SEM. P values<0.05 were considered statistically significant.

Paper I: ANOVA followed by Fisher’s PLSD was used for comparisons between control and exposed groups. The Bliss independence criterion (Bliss, 1939; Greco *et al.*, 1995), followed by Student *t*-test was applied when analyzing possible additive, synergistic or antagonistic effects of co-exposure to MeHg and PCBs (Goldoni & Johansson, 2007; Greco *et al.*, 1995).

Papers II and III: ANOVA followed by Fisher's least significant difference (LSD) or Bonferroni *post-hoc* tests were used for comparisons between control and exposed groups. For comparison of two groups student's *t*-test was applied. *P* values<0.05 were considered statistically significant.

Paper IV: Results from the behavioral tests were analyzed separately for PFOS or PFOA-exposed animals and their respective controls using two-factor ANOVA with exposure and sex as factors (predictors). For tests based on sequential measurements, such as novelty-induced activity, adaptation to a novel environment in the TraffiCage system, and accelerating rotarod, we used repeated measures ANOVA, followed by Fisher's LSD *post-hoc* test. Data were presented as mean ± SEM. *P* values<0.05 were considered statistically significant.

4 RESULTS AND DISCUSSION

In this thesis we have investigated the neurotoxicity of environmental contaminants namely PCB 126, PCB 153, PCB 180, PFOS, PFOA, and MeHg using different *in vitro* and *in vivo* experimental approaches. In *Paper I* we exposed the mouse hippocampal cell line, HT22 to different micromolar concentrations of PCB 126, PCB 153 or MeHg. We assessed cell death as the main endpoint and investigated related possible mechanisms. In *Papers II and III*, we investigated the mechanisms by which nanomoloar concentrations of PCB 153, PCB 180, MeHg or PFOS influence the proliferation or the spontaneous differentiation of NSCs. In *Paper IV*, we investigated the effects of prenatal exposure to PFOS or PFOA on mouse behavior.

4.1 INDUCTION OF CELL DEATH AND ITS MECHANISMS (*PAPER I*)

We studied the cytotoxic effects of different concentrations of MeHg, PCB 153, or PCB 126 in the HT22 cells after 4, 12, and 24 h exposures. We found that exposure to micromolar concentrations of PCB 153, 126 or MeHg induced both apoptosis and necrosis with typical morphological and biochemical alterations. Apoptotic HT22 cells exhibited nuclear condensation with PS externalization, as visualized by Hoechst 33342 and Annexin V vital staining. Necrotic HT22 cells, with damaged plasma membranes, were detected by vital staining with PI. The total cell number was also decreased. Exposure of HT22 cells to 2–4 µM MeHg, 100–200 µM PCB 153, or 25–50 µM PCB 126 for 4–12 h induced very low percentages of either apoptotic or necrotic cell death. Conversely, longer exposure (24 h) resulted in a significant increase of cell death, both apoptosis and necrosis. In comparison to our study, exposure of the AtT20 pituitary cell line to the same PCBs congeners (PCB 126 or PCB 153) for 24 h induced mainly necrosis (Johansson *et al.*, 2006), while exposure to 100 µM PCB 153 induced apoptosis in primary cortical neurons after only 4 h (Sanchez-Alonso *et al.*, 2004). This suggests that the type of cell death induced by PCBs depends on the cell type, while taking into account that late apoptosis may also display features of necrotic cell death. Induction of cell death in HT22 cells at later time point indicated that these neurons have protective capability to buffer cytotoxic effects induced by PCBs or MeHg when the duration of the exposure is short. Longer exposure duration resulted in amplified insult which was beyond the threshold of endurance of the cells leading to activation of the cell death machinery.

4.1.1 Perturbations of mitochondrial functions

The mitochondria, the power plant of the cell, have been proven to play a critical role in regulating cell death. To assess mitochondrial function, we measured the intracellular Ca^{2+} buffering capacity, the mitochondrial membrane potential, the release of cyt *c* from the intermembrane space, as well as the intracellular ATP concentration.

4.1.1.1 Intracellular Ca^{2+} buffering

Addition of Ca^{2+} to digitonin-permeabilized control cells caused a rapid increase in the concentration of Ca^{2+} in the incubation medium followed by accumulation of Ca^{2+} in

the mitochondria and restoration of the initial level. The ability of the mitochondria to accumulate Ca^{2+} was decreased at 6 h in MeHg-exposed cells and already at 2 h in PCB 153- or PCB 126-exposed cells. The Ca^{2+} buffering capacity declined to almost zero in cells exposed to the highest tested concentrations of all three toxicants. These results suggest that PCBs and MeHg caused disruption to the mechanisms that regulate homeostasis of intracellular Ca^{2+} in HT22 cells by depleting the mitochondrial capacity to buffer high calcium concentration in the cells. Similarly, exposure of SK-N-MC neurons to Styrene 7,8-oxide resulted in lower Ca^{2+}buffering capacity of mitochondria together with loss of mitochondrial membrane potential (Daré *et al.*, 2004). Taken into consideration the importance of maintaining intracellular concentration of Ca^{2+}, disturbance of Ca^{2+} regulation can compromise neuronal functions and survival. Noteworthy, besides mitochondria, the smooth endoplasmic reticulum also plays a significant role in Ca^{2+} homeostasis (Roos *et al.*, 2012).

4.1.1.2 Mitochondrial membrane potential and cyt c release

Permeabilization of the (outer mitochondrial membrane) OMM will result in the release of pro-apoptotic proteins from the mitochondria to the cytosol. We used TMRE staining to interrogate the mitochondrial membrane potential in live cells. TMRE is a cell permeable, positively-charged, red-orange dye that readily accumulates in active mitochondria due to their relative negative charge. Depolarized or inactive mitochondria have decreased membrane potential and fail to sequester TMRE. After 16 h exposure to MeHg, HT22 cells showed loss of mitochondrial membrane potential detected by the lack of mitochondrial TMRE staining in cells with condensed nuclei, while no effects were seen in PCBs-exposed cells. Supporting our results, increasing evidence indicates that MeHg induced neuronal cell death is associated with a severe impairment of intracellular Ca^{2+} homeostasis (Fahrion *et al.*, 2012; Marty & Atchison, 1998). To further examine alterations in mitochondrial function, we investigated cyt *c* release into the cytosol. The release of cyt *c* from the mitochondria plays a critical role in the activation of caspase-dependent (Li *et al.*, 1997) and caspase-independent cell death (Volbracht *et al.*, 2001). Exposure to 4 µM MeHg for 16 h results in translocation of cyt *c* into the cytosol whereas in control cells the cyt *c* showed fine networks localized in the mitochondria. In agreement with our results, exposure of neurons (differentiated from murine embryonal carcinoma cells) to MeHg compromised mitochondrial functions with subsequent release of cyt *c (*Polunas *et al.*, 2011*).*

4.1.1.3 Reduction of intracellular ATP

Disruption of mitochondrial membrane potential may affect mitochondrial functions, such as protein import and ATP production (Voisine *et al.*, 1999). Ca^{2+} play a key role in ATP synthesis by regulating vital enzymes of Krebs cycle, such as pyruvate- and α-ketoglutarate- dehydrogenases (Roos *et al.*, 2012). Moreover, changes in mitochondrial occurring during cell death require an elevated influx of Ca^{2+} into matrix (Kruman & Mattson, 1999; Lemasters *et al.*, 2002). Intracellular ATP-concentration determines the onset of either apoptosis or necrosis, where ATP concentrations between 30 and 50% of the control levels are associated with intermediate forms of cell death in the lymphoid Jurkat cell line (Leist *et al.*, 1997). In HT22 cells, exposure for 24 h to either

three toxicants (PCB 126, PCB 153 or MeHg) caused a significant depletion of ATP to 50–60% of the control level. Since neurons have small energy reserves, the ATP-thresholds determining apoptosis or necrosis might be different from other cell types. Apoptosis and necrosis can occur simultaneously in cell culture exposed to the same stimulus and often the intensity of the insult will determine the prevalence of either apoptosis or necrosis. Depletion of essential energy caused perturbation in metabolic functions and led to the onset of apoptosis and necrosis in the HT22 cells.

4.1.2 Activation of different proteases leading to cell death

4.1.2.1 Activation of calpains

To further clarify the mechanisms involved in induction of cell death in HT22, we investigated calpains and caspases. Calpains and caspases are cysteine proteases that can be activated during apoptosis, but their functions and cleavage specificities are different. We assessed cleavage of the cytoskeletal protein α-fodrin, a substrate for both calpains and caspases. Activation of calpains will result in cleavage of α-fodrin at 150 kDa fragment, while cleavages by caspases results in 120 kDa fragment (Nath *et al.*, 1996). Analysis by immunoblotting of HT22 cells revealed that the 150 kDa breakdown product increased in cells treated for 24 h. Both MeHg and PCBs have been reported to disrupt intracellular Ca^{2+} levels in different *in vitro* models (Kang *et al.*, 2004; Kodavanti & Tilson, 2000; Levesque *et al.*, 1992; Marty & Atchison, 1997). Detection of calpains, calcium-regulated proteases, after exposure to PCBs or MeHg showed that both PCBs and MeHg induced elevation of intracellular Ca^{2+} in HT22 cells. While, we only detected a small increase in the 120 kDa fragment, and accordingly no significant activation of caspases was observed at 24 h or even at earlier time points. In agreement, the pan-caspase inhibitor z-VAD-fmk could not protect HT22 cells against MeHg, PCB 153 or PCB 126 toxicity. These results confirmed that PCB- and MeHg-induced cell death is caspase-independent. In agreement with our study, chronic exposure of SH-SY5Y cells to the PCB mixture Aroclor 1254 also caused cell death via induction of calpains, but not caspase-3 (Formisano *et al.*, 2011). However, it is known that concurrent activation of calpains and caspases during induction of apoptosis can also occur (Tofighi *et al.*, 2006; Yoneyama *et al.*, 2009).

4.1.2.2 Disruption of lysosomal integrity

In cells, lysosomes play a role to store a wide range of hydrolytic enzymes that are capable of degrading macromolecule and to accumulate chemical contaminants that are damaging the cells. Lysosomal integrity may be disrupted by different toxic stimuli, leading to lysosomal rupture and leakage of lysosomal enzymes (Brunk *et al.*, 1997). Moderate stress triggers a limited release of lysosomal enzymes into the cytoplasm leading to apoptosis, while severe stress triggers a rougher release of lysosomal enzymes leading to necrosis (Brunk *et al.*, 1997). Control HT22 cells stained with the lysosomotropic dye AO revealed distinct lysosomal organelles as red fluorescence compartments under confocal microscope. Exposure to 4 µM MeHg, 200 µM PCB 153 or 50 µM PCB 126 for 16 h, caused a decrease in the number of intact lysosomes, visualized by a reduced uptake of AO and an increased green cytosolic and nuclear

fluorescence. Similarly, Dare and colleagues also found that exposure of human astrocytoma D384 cells to MeHg resulted in disruption of the lysosomal membrane in association with induction of oxidative stress (Daré *et al.*, 2001). Our results pointed to a concomitant activation of both calpains and lysosomal proteases in induction of both apoptosis and necrosis cell death.

4.1.3 Protection by protease inhibitors and antioxidants

To further confirm the involvement of calpains and lysosomal proteases in mediating toxicity in HT22 cells after exposure to PCBs or MeHg, we treated the cells with inhibitors. Incubation of the cells with the calpains specific inhibitor PD 150606 (100 µM) or the cathepsin D inhibitor Pepstatin (100 µM) prior exposure to toxicants resulted in significantly decreased percentage of dead cells. Treatment of the cells with a combination of inhibitors (PD 150606 + Pepstatin) resulted in even greater protection against both apoptotic and necrotic cell death. Pre-incubation with the cysteine protease inhibitor E64d (25 µM), an inhibitor of both calpains and cathepsins, gave similar protective effects. In our experimental model calpains and lysosomal enzymes seem to work in parallel, since pre-incubation of the cells with the calpain and cathepsin D inhibitors together resulted in significant protection from cell death.

PCBs and MeHg have been shown to induce overproduction of reactive oxygen species which results into oxidative stress in different experimental models (Aschner *et al.*, 2007; Farina *et al.*, 2011; Lee *et al.*, 2012; Selvakumar *et al.*, 2012). The antioxidant, MnTBAP is a cell-permeable superoxide dismutase (SOD) mimetic and peroxynitrite scavenger at 3–100 µM (Szabo *et al.*, 1996). It also possesses catalase activity at 10–100 µM (Day *et al.*, 1997). When HT22 cells were pre-incubated with MnTBAP (100 µM), a significant protection could only be detected in MeHg exposed cells. Similar results were obtained with the radical scavenger and glutathione precursor NAC (10 mM). These results revealed that in our experimental model, oxidative stress play a major role in neurotoxicity induced by MeHg, but not by PCBs.

4.2 EFFECTS ON NSC DIFFERENTIATION (*PAPERS II AND III*)

To investigate the effects of nanomolar concentrations of PCBs, MeHg and PFOS on the fate of NSCs during spontaneous differentiation process, we used embryonic NSCs, a model that we have implemented in our lab to study neurodevelopmental toxicity *in vitro*. Importantly, the level of exposures used were in the range found in human populations (Björnberg *et al.*, 2005; Ehresman *et al.*, 2007; Fromme *et al.*, 2007; Guvenius *et al.*, 2003).

4.2.1 NSCs proliferation

We measured proliferation of NSCs exposed to PCBs, MeHg (*Paper II*), or PFOS (*Paper III*) 7 days after inducing spontaneous differentiation by growth factor withdrawal. In *Paper II*, we found that PCBs exposure resulted in decreased cell proliferation, as detected with FUCCI transfection and EdU incorporation, and decreased the total cell number. In contrast, MeHg increased NSCs proliferation, as detected by FUCCI transfection, EdU incorporation and by Trypan blue staining. To

investigate whether the effects of PCBs are consistently detectable *in vivo*, we assessed EdU incorporation in the brains of mice prenatally exposed to PCB 153. Pregnant dams were exposed to 1 mg/kg PCB 153 in the food between E7 and E14. The dams were injected with EdU (50 mg/kg *i.p.*) 1 h prior to sacrifice. The embryos were removed at E12.5 and E14.5 and fixed by immersion in PF. In agreement with the *in vitro* studies, we found that the rate of EdU incorporation was lower in PCB 153-exposed embryos than in controls at E14.5, but not at E12.5 (Figure 4.1). The effect of PCBs was detectable at E14.5, but not at E12.5 probably because the rate of differentiation in neuronal progenitor cells is very low at E12.5 compared to E14.5 (Nowakowski *et al.*, 2002). In contrast, the effect of PCB 153 became detectable at E14.5, when the differentiation rate had increased considerably, and the change in number of proliferating cells is the cumulative outcome of increasing the number of cells leaving the cell cycle. This result is supported by our finding that PCB 153 promotes neuronal differentiation in NSCs (*Paper II*).

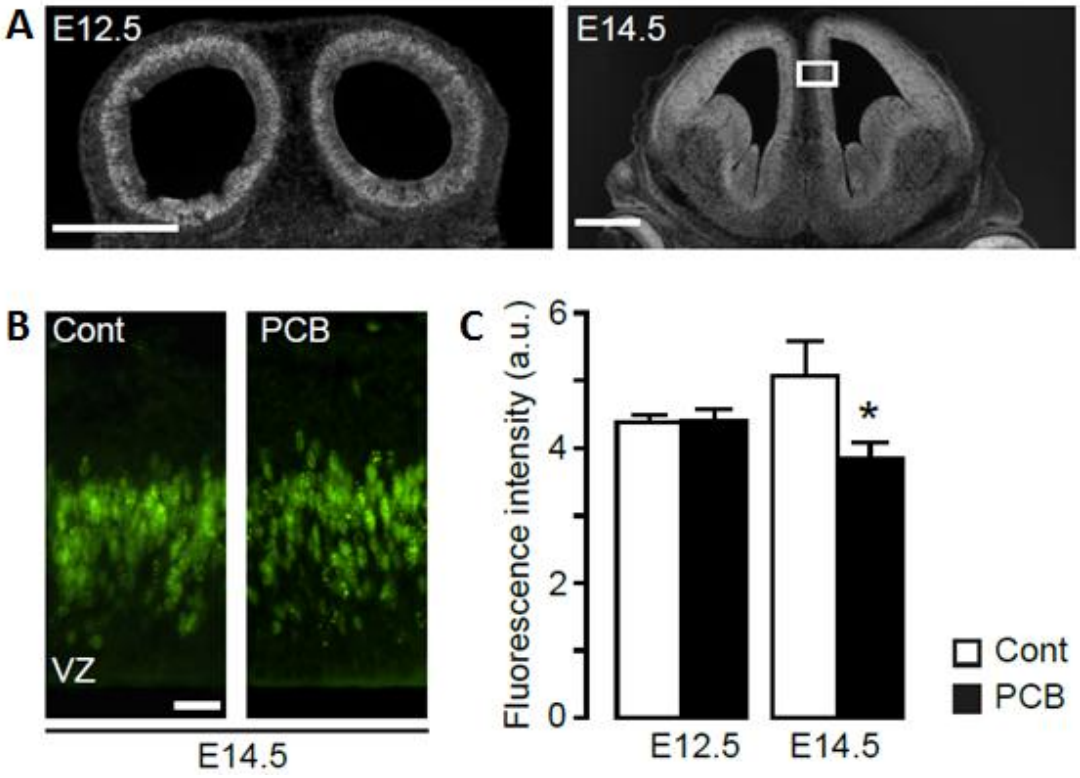

Figure 4.1 EdU incorporation in E12.5 and E14.5 mice brain (**A**). Representative visualization of EdU incorporation in E14.5 brain (**B**) and measurement of fluorescence intensity in E12.5 and E14.5 brain (**C**).

In *Paper III*, we tested a range of PFOS concentrations varying from 12.5 nM to 100 nM. PFOS induced a decrease of total cell number in a dose dependent manner. The highest concentration tested in this study (100 nM) caused a significant increase in apoptosis, while the lowest concentration (12.5 nM) did not induce any apparent changes in proliferation or cell death. Instead, exposure to PFOS at 25 or 50 nM decreased NSCs proliferation as shown by decreased number of the EdU-positive cells without affecting the percentage of cells undergoing apoptosis. Based on these facts, we defined the effects of PFOS exposure at the above mentioned doses as sub-toxic (not

leading to overt cell death), and we selected this exposure range for further studies. Our findings suggested that the decrease in the cell number registered after the current exposures could most likely be due to inhibition of the self-renewal capability. The presumable NSCs specificity of this effect is also supported by the report of Slotkin et al., who did not find any reduction in cell number in PC12 cells even after 6 days exposure to PFOS (Slotkin *et al.*, 2008).

4.2.2 NSCs spontaneous differentiation

The effects of PCBs, MeHg or PFOS on the fate of NSCs during spontaneous differentiation was further assess by performing immunostainings with the neural stem marker Nestin, early neuronal marker Tuj1, and the glial marker GFAP. In *Paper III*, we also immunostained differentiated NSCs with the oligodendrocyte marker CNPase.

In *Paper II* we found that exposure to 100 nM PCB 153 or 180, but not lower concentrations, resulted in a significant increase in the number of neurite-bearing Tuj1-positive cells compared to control cells. Fritsche et al. showed disruption of fate determination in HNP cells exposed to PCB 118 (Fritsche *et al.*, 2005), showing that the PCB leads to an increase of oligodendrocytes formation in a dose dependent manner. MeHg decreased significantly the number of Tuj1-positive cells as previously shown by Tamm and colleagues (Tamm *et al.*, 2006). Concomitantly, PCBs significantly decreased the number of Nestin-positive cells, while MeHg increased it. No significant changes in the number of GFAP-positive cells were observed in any of the treated groups as compared to their controls. In *Paper III*, exposure of NSCs to 25 nM PFOS significantly increased the number of Tuj1-positive cells, while exposure to 50 nM not only increased the number of Tuj1-positive cells, but also significantly increased the number of CNPase-positive cells. Considering the sequential differentiation of NSCs, where neurogenesis precedes gliogenesis (Qian *et al.*, 2000), the increase of differentiation in NSCs towards neuronal and oligodendrocytic cells after exposure to 50 nM PFOS is intriguing. To determine whether exposure to 50 nM PFOS influenced neurite outgrowth in Tuj1-positive cells we measured the length of the neurites and found that total neurite length was significantly increased compared to control. We also measured the maximum distance reached by the arborization of CNPase-positive cells and found that 50 nM PFOS significantly increased the number of CNPase-positive cells with further distance of arborization processes compared to control cells. In addition, PFOS exposure decreased the expression of Nestin, but had no effect on the number of GFAP-positive cells as compared to controls.

4.2.3 Functional readout of NSCs differentiation

Spontaneous Ca^{2+} oscillations have been implicated in different vital cell processes such as progression of the cell cycle, regulation of migration and neuronal differentiation (Gomez *et al.*, 1995; Gu & Spitzer, 1995; Komuro & Rakic, 1996; Resende *et al.*, 2010). Spontaneous Ca^{2+} signals have been shown to be more frequent at early stages of neural precursor differentiation and become less frequent as the stem cells differentiate into mature neurons (Ciccolini *et al.*, 2003). Thus, spontaneous Ca^{2+} activity can be used as a functional readout to assess the state of differentiation in NSCs. In *Paper II*, we measured spontaneous Ca^{2+} oscillations after 7 days of

spontaneous differentiation. We found that the number of cells showing spontaneous Ca^{2+} activity was decreased by PCBs, and increased by MeHg. Differentiated neurons respond to glutamate, therefore we also examined the response of NSCs to glutamate by directly adding it in the culture medium of the control and exposed cells. Both PCBs increased the number of glutamate-responsive cells, but statistical significance was reached only in the PCB 153-exposed NSCs. MeHg significantly decreased the number of glutamate-responsive cells in agreement with the observed decrease of neuronal differentiated cells suggesting that the MeHg exposed cells were less differentiated compared to PCBs exposed cells. In *Paper III*, we found that exposure to 25 nM or 50 nM PFOS decreased the number of cells showing spontaneous Ca^{2+} activity compared to the control cells, confirming that PFOS-exposed cells were more differentiated compared to control cells.

4.2.4 Signaling pathways

To elucidate the mechanisms involved in changing the fate of NSCs during spontaneous differentiation after exposure to PCBs, MeHg or PFOS, we checked the mRNA expression of Notch signaling target genes, Hes5 and Math1 (*Paper II*) and PPAR genes and their target genes, UCP2 and 3(*Paper III*). In *Paper II* we also measured the level of Notch1 using western blot. We found that PCBs-exposed cells showed higher levels of Notch1, whereas MeHg-exposed cells showed lower levels. We then investigated the expression of downstream genes, Hes5 and Math1, anti-neuronal and pro-neuronal transcription factors respectively. After 3 days of spontaneous differentiation, we found a significant downregulation of Hes5, and an upregulation of Math1 in PCB 153- and 180-exposed cells. In an earlier report, exposure to the PCB mixture Aroclor 1254 increased the expression of Hes1 and Hes5 in the fetal rat brain. The apparent contradiction may be explained by the timing of analysis of Hes expression, as well as by the presence of different PCBs in the mixture (Bansal *et al.*, 2005). Conversely, in MeHg-exposed cells we found a significant upregulation of Hes5, and a downregulation of Math1. We then further analyzed the relevance of the Notch signaling by blocking the cleavage of Notch receptors with the γ-secretase inhibitor DAPT (2.5 mM) administered daily to NSCs undergoing spontaneous differentiation and found that DAPT only prevented the effects of MeHg. This supports the hypothesis that PCBs exert a repressive action on Notch signaling, while the effects of MeHg are mediated by activation of Notch signaling. Our results on MeHg are consistent with the findings by Tamm and colleagues (Tamm *et al.*, 2008).

Since PFOS has been shown to exert its effects via PPAR signaling in different experimental models (Fang *et al.*, 2012; Takacs & Abbott, 2007; Vanden Heuvel *et al.*, 2006), we checked the possible involvement of PPAR signaling in fate determination NSCs (*Paper III).* In contrast to earlier reports (Jacquet *et al.*, 2012; Shipley *et al.*, 2004), we found no changes in the expression of PPARα or PPARδ isoforms. Instead, we detected a significant upregulation of PPARγ mRNA after exposure to 50 nM PFOS. Exposure to the same concentration of PFOS leads also to an upregulation of the mitochondrial uncoupling proteins UCP2, a downstream gene under the control of PPARs (Kelly *et al.*, 1998; Villarroya *et al.*, 2007). Exposure of NSCs to PPARγ agonist, RGZ increased the expression of PPARγ and UCP2, and also increased

neuronal and oligodendrocytic differentiation. To further understand the role of PPARγ in our experimental model, we pretreated the primary culture of NSCs with GW9662 and found that it blocked the effects of PFOS on differentiation. These results confirmed that PPARγ activation mediates the effects of PFOS neuronal and oligodendrocytic differentiation of NSCs. Neuronal or oligodendrocyte commitment has been associated with PPARγ activation in different cell models (Bernardo *et al.*, 2009; Park *et al.*, 2004; Sim *et al.*, 2008).

To check whether the effects on PPARs and UCPs expression are consistent *in vivo*, we measured their mRNA expression in cortical tissues from newborn mice exposed to 0.3 mg/kg/day PFOS throughout pregnancy. Interestingly, we found that prenatal exposure to PFOS also upregulated PPARγ and UCP3. These results indicate that PFOS might exert its toxic effects by activating the same signaling pathway *in vivo* as *in vitro*.

4.2.5 Effects of combined exposure

Since both PCBs and MeHg can be found in the same food sources and have some common sites of actions, we investigated the effects of simultaneous exposure to micromolar (*Paper II*) and nanomolar (*Paper III*) concentrations of MeHg and PCBs. In *Paper II*, the results suggested an antagonistic interaction in most cases, particularly with regard to necrotic cell death. Interestingly, we observed a synergistic interaction between the highest concentration of MeHg and PCB 153 in relation to the induction of apoptotic cell death. In *Paper III*, we evaluated the effects of combined exposure on spontaneous neuronal differentiation. Neither toxicant induced cell death in NSCs when applied alone in the selected concentrations. However, the exposure to both combinations (PCB 153+MeHg or PCB180+MeHg) significantly increased the number of cells undergoing apoptosis. Conversely, the effects of the combined exposures on neuronal differentiation pointed to an antagonistic interaction between PCBs and MeHg. *In vitro* and *in vivo* studies on combined exposure to PCBs and MeHg have reported antagonistic or synergistic interactions, as well as additive effects (Bemis & Seegal, 2000; Costa *et al.*, 2007; Johansson *et al.*, 2006; Piedrafita *et al.*, 2008), and the results appear to depend on the experimental model, parameters considered, and modality of exposure. Interestingly, a cohort study from the Faroe Island showed no PCB-effects in children with low mercury exposure, while children with high mercury showed PCB-associated neurobehavioral deficits pointing to a possible interaction between the two neurotoxic agents (Grandjean *et al.*, 2001). Considering the relevance of combined exposures to environmental contaminants, further studies are needed to clarify the mechanisms involved in these complex chemical/biological multiple interactions.

4.3 DEVELOPMENTAL NEUROTOXICITY OF PFCS *IN VIVO* (*PAPER IV*)

In *Paper IV*, we exposed pregnant mice to PFOS or PFOA (0.3 mg/kg) from GD1 throughout pregnancy. We found that dams exposed to PFOS or PFOA gained weight normally during pregnancy and did not differ from control females at any gestational age. Litter size and sex ratio were similar in control and exposed groups. There were no differences in offspring body or brain weights between groups at birth. Liver weights were normal in PFOS exposed pups, but significantly increased in PFOA-exposed

mice. Levels of PFOS or PFOA in control samples were below detection limit. Prenatal exposure to the same dose of the chemicals (0.3 mg/kg, GD1–20) resulted in a lower liver level and higher brain concentration of PFOS as compared to PFOA.

4.3.1 Behavioral alterations

The effects of prenatal exposure on the offspring were investigated in a battery of behavioral test to evaluate motor function, circadian activity and emotion-related behaviors.

First we investigated the **motor function** in the open field, hanging wire and accelerating rotarod tests. We found that PFOS exposure had a significant effect predominantly in males (significantly shorter distance covered, shorter latency to fall, and shorter time on the accelerating rotarod during the last trial), while the females had only shorter latency to fall from the accelerating rotarod during the first and the last trials (although they appeared to be able to acquire the motor task as efficiently as the controls). In contrast, PFOA exposure had significant effect only in females in the accelerating rotarod test, where they performed worse than the controls in every trial, but the differences reached statistical significance only in the third trial.

To further investigate the toxic effects of PFCs, we measured **circadian activity** of mice housed in social groups by using the TraffiCage™ system. Novelty of the environment evoked increased exploratory activity in all groups lasting 2–3 h. Analysis of the activity during the first 3h after putting the mice in a new cage revealed effects that depended on both sex and exposure. Thus, PFOS-exposed males displayed decreased activity during the first 2 h. A similar trend was observed in PFOS-exposed females, but the difference did not reach significance. In the PFOA groups, activity levels differed during the first hour of the test in a sex-related manner. PFOA-exposed males were more active, while PFOA exposed females showed a decreased activity compared to controls. After habituation to the new home cage, animal activity declined to a low, diurnal level. All groups of animals had a normal circadian pattern with higher levels of activity during the dark phase and early morning hours, followed by lower activity levels during the light phase. There was no significant difference in total activity counts over light or dark periods between control and PFOS-exposed groups, either in males or females. Activity of PFOA-exposed males was higher than in controls, especially during the dark phase. Total activity counts were also higher during the light phase in PFOA-exposed male group due to more prominent activity peaks in the morning hours. PFOA-exposed females did not differ significantly from controls.

The signs of altered locomotor activity in the exposed groups prompted us to extend the analysis of behavioral data by calculating resting time and its circadian distribution over the second day of the experiment. Interestingly, we found no significant differences between any groups in total resting time (data not shown), but in the number of inactive periods. We found that control females consistently had less inactive periods than control males during the dark phase. Exposure to PFOS caused an increase in the total number of inactive periods in both males and females, with differential increase for both light and dark phases only in the females. The increased number of inactive period suggested that PFOS exposed mice become fatigued faster

than controls. PFOA exposure had an opposite effect decreasing the total number of inactive periods in both males and females. The effect was significant during the light phase in both sexes; in addition, in males, the decrease was significant also in the dark phase of the circadian cycle.

Altogether, the results showed that offspring exposed to a low dose of PFCs *in utero* exhibit altered locomotor activity level and circadian distribution, impaired muscle strength and poor motor coordination, as well as decreased exploratory activity. Interestingly, the outcome of prenatal exposure to different PFCs appears to be sex- and chemical-dependent. These effects are more pronounced in PFOS-exposed males, while in females they either attenuated or absent. Prenatal exposure to PFOA induces opposite effects on exploratory behavior in male and female offspring, as well as elevated activity of males in the home cage.

We also investigated **anxiety-related behavior** in the exposed offspring. To this end we used elevated plus maze and found significant differences only in PFOS-exposed males. Thus, PFOS-exposed males spent significantly more time inactive, as well as less time in the open arms. However, the ratio between open and closed arms visits was not altered (around 60:40%), therefore the preference for exploration of open (potentially dangerous) versus closed (safe) areas did not seem to be altered. In agreement with the globally lower level of locomotion described above, the total distance walked was shorter. PFOS exposed females as well as all PFOA-exposed groups did not differ from their respective controls in any parameter measured in the elevated plus maze. To assess possible **depression-like behavior**, we used the forced swimming test and we found that neither exposure, nor sex had a significant effect on immobility time. In summary, these results indicate that prenatal exposure to PFCs did not induce anxiety-like behavior or depression-like behavior in the offspring.

The mechanisms behind the neurobehavioral effects of PFCs are not understood yet. Hormonal alterations due to endocrine disruption potentially induced by PFCs may play a critical role, but further studies are needed to confirm this hypothesis.

4.3.1.1 Preliminary behavioral data on zebrafish

We also tested the effects of PFOS on zebrafish as additional animal model that we have recently implemented in our laboratory. It is often difficult to reproduce *in vitro* results *in vivo* mostly due to pharmacokinetics. Zebrafish provide a cost-effective model to bridge the gap between *in vitro* and *in vivo* because it offers an advantageous and very controllable route of administration of toxicants during development (dissolved in the rearing water). In addition, behavioral effects can be assessed very early in development (as early as 4 dpf for VMR), and early alterations appear very stable with age (Levin *et al.*, 2003).

We exposed wildtype AB zebrafish embryos to 0.1 mg/l PFOS in a static, non-replacement regimen starting from 2 hour post fertilization (hpf) until the behavioral testing. The larvae were plated at 2 hpf and maintained individually in 48-well plates in E3 water for the entire duration of the experiment at 28^{o}C in a 14:10 h light-dark cycle (light on at 9 a.m.). We assessed the VMR at 4 dpf in alternating light and dark

(3x10:10 min) in an automated videotracking system (Noldus, Wageningen, The Netherlands). The total distance moved was analysed for the last dark period.

The results from PFOS exposure in zebrafish larvae were compared with the locomotor activity in PFOS exposed mice and their controls during the first 2 h after placing in a new cage (see also *Paper IV* for details). We have used 2 independent parameters to characterize the activity during the observation period: the total distance moved (swam or walked, respectively), and the index of curvature (IOC). The IOC is a synthetic descriptor of the shape of locomotor activity curve against time, and it measures the degree and direction of deviation from a flat distribution of activity within a limited observation period (Fry *et al.*, 1960). A faster decay of the level of activity yields a more negative IOC. We found that developmental exposure to PFOS results in lower total activity associated with a larger negative IOC in both species. This can be interpreted as fatigue developing faster in PFOS-exposed animals. This interpretation is supported by the performance of PFOS-exposed mice in the accelerated rotarod test (*Paper IV*). Further support comes from the finding that developmental exposure to PFOS induces alterations in the developing peripheral nervous system in zebrafish larvae (Zhang *et al.*, 2011). These results are therefore promising for the intended use of zebrafish larvae as a primary screening for behavioral alterations induced by chemicals with potential developmental neurotoxicity.

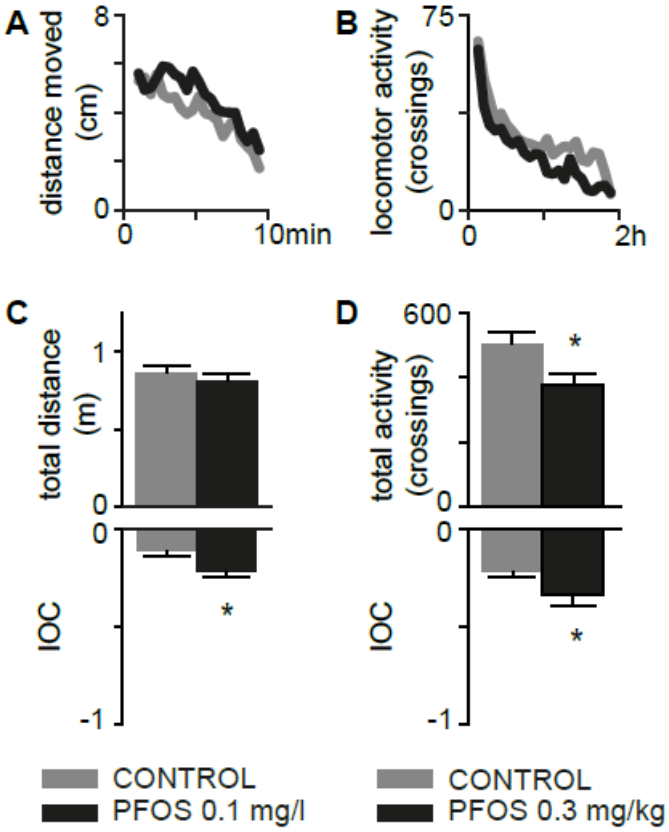

Figure 4.2 Comparison of activity of zebrafish (**A, C**) and mice (**B, D**). (**A)** VMR in 4 dpf zebrafish larvae during 10 min dark pulse. (**B**) Hyperactivity induced in mice in a new homecage. (**C, D**) Developmental exposure to PFOS results in decreased total activity associated with more negative IOC, suggesting faster development of fatigue in both species.

4.4 GENERAL CONSIDERATIONS

The presence of chemicals yet untested for developmental neurotoxicity is a matter of major concern. Accurate precautionary regulation of environmental contaminants may provide better protection for child development. Publicly available information on neurodevelopmental toxicants could contribute to increasing public awareness and thereby reduce the exposure. Numerous evidences indicate that PCBs and MeHg exposure to developing nervous system contributes to behavioral alterations in children. We investigated the effects of known developmental neurotoxic agents, PCBs and MeHg at cell death and differentiation level to understand the underlying mechanisms of neurotoxicity possibly explaining the effects seen in general population. In addition we investigated a potentially neurotoxic environmental contaminant, PFOS, and found that it influences a fundamental developmental process – the differentiation of NSCs. Moreover, we found that the effects found *in vitro* are reproducible (to a certain extent) *in vivo*, and we also found behavioral alterations in mice and zebrafish. Importantly, the effects of all food contaminants tested are mediated by alterations in signaling pathways (Notch and PPAR) which are fundamental for development and physiological functions. Another relevant aspect is that the effects of combined exposure cannot be directly inferred from single exposure data. Considering that humans are most often exposed to cocktails of environmental and food contaminants, a combined exposure approach becomes more relevant for predicting possibly more severe neurotoxic effects in target populations.

Multiplicity of adverse effects induced by neurotoxic agents alone or in cocktail confounds the knowledge on the mechanisms of neurotoxicity. *In vitro* tests provide results faster compared to *in vivo* testing, which is proven to be more expensive, time- and labor-intensive, and requires a large number of animals. It is important to bear in mind that *in vitro* experiments could be less precise than *in vivo* experiments because they do not reproduce the actual conditions in the whole organism. When comparing the environmental level of exposure with the *in vitro* doses, one should consider several factors intrinsic to the experimental models, such as the cell type (cell line or primary cultures), the cell density, the volume and composition of cell culture media, binding of the chemicals to the walls of culture flasks, and the potential masking properties of serum (Meacham *et al.*, 2005). For instance, serum albumin inhibits the toxic effects induced by PCBs (Lee *et al.*, 2001), an effect that we have also observed in our experimental models (unpublished observations).

Cell lines appear to be a relevant model for *in vitro* screening of neurotoxic agents and dissecting the mechanisms of neurotoxicity in differentiated cells, while NSCs appear to be a relevant model for *in vitro* developmental neurotoxicity studies. Endpoints such as cell death, cell proliferation and differentiation appear to be reliable, depending on the cell type. It is therefore important to use multiple cell models for *in vitro* neurotoxicity assessments. The implementation of appropriate *in vitro* models and methods as part of integrated test strategies incorporating mechanistic assays could accelerate the process of chemical testing. In addition, the identification of relevant mechanisms may lead to novel preventive and protective strategies, and reduce and refine the use of animals (Coecke *et al.*, 2007; Lein *et al.*, 2007). However, it may be

too ambitious to think that *in vitro* methods could completely replace the *in vivo* testing methods for assessing developmental neurotoxicity of chemicals. The complexity of the nervous system requires that functional analyses are included in the assessment. Behavioral testing offers vast possibilities because it allows a non-invasive functional evaluation, and may provide information on the neuroanatomical or neurochemical system(s) damaged. The implementation of zebrafish as a complementary model for developmental neurotoxicity is a promising approach, yet further research is warranted for validation of this model.

5 FUTURE PERSPECTIVES AND CONCLUSION

In response to a substantial increase of neurodevelopmental disorder in children together with higher number of chemicals been produced every year, and the awareness that huge resource requirements of animal-base models of toxicity testing, the push for new testing methods has focused on developmental neurotoxicity testing. The motivation to develop alternative /complementary methods for assessing developmental neurotoxicity embraces not only the promotion of humane science according to 3R principles, but also the consciousness that traditional, animal-based models of toxicity testing will not be able to keep up with the high demands of modern society. An efficient testing method is compulsory to make sure that the government and legislation committee can provide guidelines about all chemicals that have been or will be released to public use. Zebrafish fit perfectly to the requirement of new testing paradigm as it can be used to replace mice for several chemical testing especially for screening purposes. Our future goal is to produce data that are reproducible and reliable so that it can be validated.

Toxic chemicals, such as highly dangerous pesticides that are banned in industrialised countries, are exported to developing societies, where environmental and occupational standards are often weak or at least poorly enforced (Eddleston *et al.*, 2002). Moreover, the consequences of chemical exposures are largely unreported. Rapid developments in industrial and agricultural sector in many countries such as Malaysia have a high impact on economy and society. A wide variety of contaminants are discharged into the environment every day from the residential, commercial and industrial sources without clear rules and regulations. The potential health impact to human and young children is neglected due to lack of awareness in the developmental neurotoxicity field. In term of rules and regulations on how to handle chemicals, Malaysia is still far away behind developed countries. Lacks of study have been conducted in the developmental neurotoxicity field probably due to low awareness about the adverse effects of developmental exposure to neurotoxicants and lack of experts in this field. My future goal is to convey the message to the government and society about the high susceptibility of the developing nervous system to chemicals as compared to adult.

The growing concern about the increasing incidence of neurodevelopmental disorders and the possible role that environmental contaminants may play as etiopathological factors call for more research in the field of developmental neurotoxicology.

A combined experimental approach integrating *in vitro* with *in vivo* models appears to be the best strategy to understand the mechanisms of neurotoxicity and identify relevant endpoints that may be used for risk assessment of potential neurotoxic substances.

The ultimate goal is to provide assistance to other federal agencies in the development of environmental standards and programs to ensure adequate protection for the young generation so that harmful effects on human health are avoided or minimized.

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